DIAGNOSTIC AND PROGNOSTIC TEST FOR PERIODONTITIS USING NUCLEAR MAGNETIC RESONANCE AND MASS SPECTROMETRY

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I. Introduction

Periodontitis' Definition and Prevalence

Periodontitis is characterized by microbially-associated, host-mediated inflammation which results in loss of periodontal attachment (**Tonetti et. al. 2018**). This is identified in the form of clinical attachment loss (CAL) in the existing dentition, measured from the cemento-enamel junction (CEJ) as a reference point to the tip of a periodontal probe during periodontal diagnostic probing. Severe periodontitis is the 6th most prevalent disease in the world (**Kassebaum 2014**). According to estimates, the prevalence of periodontitis during the years 2009-2014 was reported as 42% for dentate American adults over 30 years old (approximately 65 million adults), with 7.8% having severe periodontitis (**Eke et. al. 2012, 2018**). The case definition of periodontitis in the above study included all cases that have ≥2 interproximal sites with CAL ≥3 mm and ≥2 interproximal sites with pocket depth (PD) ≥4 mm (not on the same tooth) or one site with PD ≥5 mm (**Eke et. al. 2012**).

However, for the purposes of this manuscript, the periodontitis case definition reported in the 2018 Workshop for a new classification of Periodontal and Peri-implant Diseases and Conditions is used: presence of interdental CAL detectible at ≥ 2 non-adjacent teeth, or buccal or oral CAL ≥ 3 mm with pocketing > 3 mm is detectible at ≥ 2 teeth (**Tonetti et. al. 2018**). Excluded are sites of recession originating from trauma, cervical dental caries, CAL on the distal site of 2nd molars potentially related to malposition or a 3rd molar extraction, an endodontic lesion that is draining through the gingival margin and the presence of a vertical root fracture.

Diagnosis and Prognosis of Periodontal Disease

Armitage in 1999 proposed a classification of periodontal diseases (**Armitage 1999**). He classified periodontitis into chronic, aggressive, periodontitis as a manifestation of systemic diseases and necrotizing periodontal disease. He subdivided chronic and aggressive periodontitis into localized and generalized and assigned three categories of severity: slight, moderate and severe. The most recent classification of periodontal conditions by the 2018 Workshop identified necrotizing periodontitis, periodontitis as a manifestation of systemic diseases and periodontitis. Periodontitis was subdivided into Stages I to IV, according to severity and according to the rate of progression into Grade A to C, thus eliminating the category of Aggressive Periodontitis of the previously mentioned classification (**Papapanou 2018**). This study focuses on severe chronic and aggressive periodontitis, according to the Armitage classification, and Stages III and IV Periodontitis, according to the Papapanou classification, with CAL loss of 5 mm or more.

Additionally, whereas several prognosis classifications exist (**McGuirre 1996, Kwok & Caton 2007, Martin 2009**), definitive and accurate predictors to integrate into the treatment plan have still not been identified (**Nunn 2012**). The high negative predictive value of bleeding on probing (BOP) – absence of BOP indicates periodontal stability – makes it a good predictor for disease progression (**Lang 1990**).

Currently, there is no accurate way to predict the initiation and progression of periodontal disease. Thus, clinicians do not differentially treat periodontitis, according to susceptibility to risk of future disease. This is inefficient and expensive as an approach and it leads to unnecessary treatment for multiple patients. In 2011 alone, the cost of periodontal treatment in the U.S. approximated 50 billion dollars (**Flemmig 2013**). In 2015, in the dental field, the greatest losses in productivity were attributed to tooth loss ($126.67

billions – 67%), with an additional $38.85 billion (21%) to severe periodontitis (**Righolt 2018**). This is the reason why it is crucial to develop a site-specific prognostic test to predict future periodontal disease initiation and/or progression.

Development of a Test in "-omics" Era

Periodontitis' diagnosis is currently based on the use of the periodontal probe which is utilized to measure the depth of dento-gingival sulcus in millimeters (mm) and the extent of CAL. This is a rather rudimentary tool, which is very uncomfortable for the patient and identifies periodontitis after it has developed. It also entails measurement error (±1mm) by the examiner (**Watts 1995**). For reference, a change of 2 mm in the depth of the pocket, which is considered disease progression in a 12-14 mm-long root of the tooth; thus, the periodontal probe's lack of consistency is emphasized. Various contemporary tests for periodontitis have been developed that measure periodontal microorganisms, proteins and enzymes in the periodontal pocket (**Armitage 2003**). While these tests may provide interesting information regarding periodontitis in a specific individual, their use in the clinical setting has not been yet validated.

The most promising field of studies for the development of an appropriate diagnostic test for periodontitis onset and progression is the "-omics" field. Also referred in the literature as "multi-omics", this field allows not only for diagnosis and detection of the disease, but also for differentiating among its various stages and determination of treatment outcomes, acting as "tools" for understanding the dynamics of periodontal disease progression (**Callif 2017**).

According to a recent review, the data obtained by the use of "-omics" technologies have great potential to inform paradigm shifts in our understanding of periodontal diseases, but data management, analysis and interpretation are to be done with systematic bioinformatics approach, to reach meaningful conclusions (**Grant 2012**). The genomic investigations aim to study the genome. They have succeeded in finding the most important susceptibility loci responsible for periodontitis. The number of genetic loci associated with periodontal disease obtained from genomic studies of aggressive and chronic periodontitis is increasing (**Morelli 2020**). Few loci, mainly identified for aggressive and severe forms of the disease, have been replicated and reached statistical significance. Most of the genomic studies in periodontitis have small or moderate sample sizes and are very heterogenous in their methods and reported results.

Along with genomics, transcriptomics aims to find specific genes and single nucleotide polymorphisms which are associated with an increased risk to develop periodontitis, as well as to detect differences in gene expression in periodontitis-affected individuals. Usually, genomic and transcriptomic studies have been focusing on one or limited candidate genes/transcripts. In other studies, genome- or transcriptome-wide approaches have been carried out, omitting an a priori positive discrimination of certain genes. These studies require the extraction of DNA or RNA from blood, epithelial cells collected from an oral swab, gingival tissue, among other sources. In these studies, classical inflammatory mediators have been identified (**Trindale 2014**).

The aim of proteomics is the evaluation of the complete protein and peptide in health and periodontitis. It includes the technological applications to identify and quantify the sum of proteins existing in cells, tissues of an organism. Proteomics is able to analyze

and categorize the overall protein signature tracing all the way back to the genome. Technological applications involving proteomics are used in many branches of research involving diagnostic markers' detection, production of vaccines, pathogenesis mechanisms comprehension, understanding of the protein pathways in various diseases (**Aslam 2016**).

The most recent studies, however, focus on metabolomics, which has not yet been studied in equal detail to the previous three branches of the multi-omics (**Veensra 2012**). Referring back to model of genomics, transcriptomics, proteomics and metabolomics, our greatest chance in finding meaningful periodontitis biomarkers will be analyses that report correlations at all four levels. Metabolomics has gained popularity since it profiles directly the phenotype and changes thereof in contrast to other "-omics" technologies (**Amberg 2017**). Metabolomics' ultimate aim is to obtain a complete screen of all the metabolites of a given biological sample and interpret how the metabolic profile changes with a given pathophysiological state.

However, the technology to study metabolomics only recently reached its peak. The main techniques to study metabolomics are Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy. NMR and MS both have their own advantages and disadvantages in conduction of metabolomic studies. MS's greatest advantage is its high sensitivity, being able to identify hundreds of species in a single sample. The test's sensitivity allows the identification of multiple compounds with rare false positive results. One of the main weakness of MS is quantification. The MS signal intensity of any compound is affected by the type of sample preparation used and efforts to correct for that can lead to inaccuracy and imprecision (**Veenstra 2012**). MS entails a high level of bias, requires multiple steps for preparation, it is only moderately reproducible and is destructive

(**Emwas 2015**). The major weaknesses of MS are the advantages of NMR. NMR is very precise and non-biased. It requires fewer steps than MS to be conducted, it is non-destructive and very reproducible. However, it has low sensitivity. It is obvious, thus, that these two procedures are completing each other's weaknesses.

Studies on the Diagnostic Value of Saliva for Periodontitis

There have been multiple studies that have investigated the existing salivary metabolites that are used as diagnostic tools. Tongue swabs and saline washouts have been also able to identify metabolites that are related to health and disease through NMR analysis (**Gawron 2019**). Additionally, the salivary metabolic fingerprints, through NMR, of chronic and aggressive periodontitis have been studied with no significant differences between the two conditions (**Romano 2018**).

Identified salivary metabolites associated with periodontal disease are the dipeptides leucylisoleucine, phenylphenol, and serylisoleucine as well as the fatty acids arachidonate, arachidate, and dihomo-linolate are attractive candidate markers (**Barnes 2011**). Another study reported increased concentration of acetate, c-aminobutyrate, n-butyrate, succinate, trimethylamine, propionate, phenylalanine and valine in chronic periodontitis subjects and reduced pyruvate and N-acetyl groups compared to periodontal health (**Aimetti 2012**).

A recent systematic review on metabolomics investigating saliva samples reached the conclusion that valine, phenylalanine, isoleucine, tyrosine and butyrate are increased in periodontitis-affected subjects, whereas lactate, pyruvate and N-acetyl groups were increased in periodontally healthy patients (**Baima 2021**).

Other salivary metabolites positively associated with increased PDs are: caproate, isocaproate/butyrate, isovalerate, isoleucine, isopropanol/methanol, 4-aminobutyrate, choline, sucrose, sucrose/glucose/lysine, lactate/proline, lactate and proline (**Garcia-Villaescusa 2018**). The bacterial metabolite phenylacetate isolated from saliva has been significantly associated with periodontitis (**Liebsch 2019**).

From the data of another systematic review, several biomarkers have been assessed in the literature for their sensitivity and specificity in diagnosing periodontitis. However, there is no assessment of sensitivity and specificity in metabolomics studies. MIP-1α, IL-6, IL-1β and MMP-8 were acceptable. The combination of IL-6 and MMP-8 showed the most promising diagnostic accuracy. However, the studies included in the literature review are very heterogenous and the results need to be cautiously interpreted (**KC 2020**).

In another systematic review and meta-analysis, MMP8, MMP9, IL-1β, IL6 and hemoglobin in saliva were indicated as the biomarkers with the ability to detect periodontitis in medically healthy subjects. The highest sensitivity to detect periodontitis was attributed to IL-1β and the highest specificity for MMP-9 81.5% (**Arias-Bujanda 2020**).

Studies on the Prognostic Value of Saliva for Periodontitis

Other studies have looked into the predictive value of salivary metabolites concerning periodontitis development and progression. Cadaverine, 5-oxoproline, and histidine have been shown to be metabolites isolated in saliva that are associated with periodontitis and could potentially predict the onset or progression of that disease (**Kuboniwa 2016**).

Furthermore, an NMR-based study showed that non-surgical periodontal treatment is able to lead to significant changes in the salivary metabolic profile of a periodontitis patient, however it will still be distinct from the one of a healthy patient (**Romano 2019, Citterio 2020**).

Studies on the Diagnostic Value of Gingival Crevicular Fluid for Periodontitis

One of the most promising oral fluid that is rich in biomarkers is the gingival crevicular fluid (GCF). The GCF is a biologic fluid and inflammatory exudate that originates from the blood vessels in the connective tissue of the gingiva, which is sub-adjacent to the epithelial lining of the dento-gingival space (**Barros et. al 2016**). The literature suggests that GCF from clinically healthy tissue is an altered serum transudate, which becomes inflammatory exudate in the presence of periodontitis and gingivitis. Additionally, GCF has a different microbial profile; the concentration and composition of molecular biomarkers between health and disease can be used to predict patient-based and site-based outcomes. Whereas, saliva is more readily available compared to GCF, the latter has the advantage that it can reveal data on the metabolic profile on periodontitis in a site-specific way. This could predict changes on a site-level that may occur to the periodontium in the future, prior to the onset or the progression of the disease, thus avoiding periodontal breakdown in terms of CAL loss, PD increase, bone loss, mobility and eventually tooth loss.

GCF has been introduced as a diagnostic aid, using its flow and contents, since the 70s (**Golub 1976**, **Smith 1977**). There are currently more than 90 elements found in the GCF that may be of use as diagnostic and/or prognostic markers. Those elements include

inflammatory mediators, markers of oxidative stress, host-derived enzymes, tissue-breakdown products and mediators of bone homeostasis (**Ghallab 2018**).

There has been a series of studies that focused on antibodies in the GCF as diagnostic biomarkers for periodontitis. It has been proven that immunoglobulins can be detected in GCF and, in certain sites, they are in even greater levels than in serum, which supports the concept of specific local antibody synthesis in gingival tissues (**Ebersole 1984, Smith 1985**). In a follow-up study of the same group, the elevated antibody levels were correlated with responses to specific periodonto-pathogenic bacteria (*Aggregatibacter actionomycetemcomitans*, *Porphyromonas gingivalis* and *Bacteroides intermedius*) (**Ebersole 1985**).

Weak association has been found between the myeloperoxidase (MOP) in the GCF and clinical measurements of periodontal disease, but the concentration of it is higher in the diseased sites; however, GCF's MPO previously observed at periodontitis sites is not specific to such sites. The increase of MPO in GCF likely occurs when additional polymorphonuclear leukocytes (PMNs) enter the sulcus as a result of gingival inflammation (**Smith 1986, Cao 1989**).

Neutrophil elastase (NE) is another enzyme that is elevated in the GCF in periodontitis patients. The increase NE available in PMNs compared to NE inhibitors in GCF suggest that there is a transient increase in free NE, which leads to tissue destruction (**Smith 1994, Smith 1995**).

The glycosamynoglycans chondroitin-4-sulphate and hyaluronan have been reported to be elevated in periodontitis and to be reduced with periodontal treatment; thus,

they may be used as diagnostic markers for periodontitis (**Smith 1995, Smith 1997**). Proteoglycans metabolism, as analyzed by the determination of uronic acids in GCF, did not seem to significantly affect clinical measurements (**Huri 2003**).

In the 1990s, Smith is the one that starts to look into GCF proteomics in a site-specific way. In a study looking at lactate dehydrogenate (LDH), aryl sulfate (AS) and NE, all varied from sampling to sampling. However, the differences among sites were retained throughout the length of the experimental period (**Smith 1991**). It seems that the previously mentioned MOP is one of the most useful enzymes as biochemical marker for severe periodontal disease in a site-specific manner (**Smith 1992**). It is important to select representative surfaces in the mouth and for that reason, multiple studies, including this one, have pre-selected the Ramfjord teeth for sampling (**Ramfjord 1967**)

Multiple studies have aimed at the characterization of the GCF proteome identifying between 199 (**Carneiro 2012**) and 327 (**Tsuchida 2012**) proteins in periodontally healthy sites. Other studies, have demonstrated a comparison between periodontally healthy and diseased sites. In a study by Kido in 2012**,** using Mass Spectrometry it was shown that 64 proteins were observed only in healthy sites, while 63 proteins were observed in diseased sites (**Kido 2012**).

From the data of a recent review, angiotensinogen, clusterin and thymidine phosphorylase were present only in “periodontal health”, whereas neutrophil defensin 1, carbonic anhydrase 1 andelongation factor-1 γ were more frequently present in “chronic periodontitis”. The number of proteins identified in GCF by proteomics may vary substantially, from dozens to hundreds, depending on the study design and statistical analysis. However, proteomics has not given definitive answers in periodontitis’

pathogenesis, other than the fact that multiple, rather than single proteins have to be considered for efficient disease characterization (**Bostanci 2018**).

As far as metabolomic studies on the diagnostic value GCF for periodontitis, the metabolic pathway of purine degradation, which is a major source for reactive oxygen species production, was significantly accelerated in periodontitis patients (**Barnes 2009**). Putrescine, lysine, phenylalanine, ribose, taurine, 5-aminovaleric acid and galactose were identified to be higher in greater PDs (**Ozeki 2016**). From cross-sectional data, the following metabolites were associated with generalized aggressive periodontitis: noradrenaline, uridine, α-tocopherol, dehydroascorbic acid, xanthine, galactose, glucose 1-phosphate and ribulose 5-phosphate (**Chen 2018**).

A more recent study uracil, N-carbamylglutamate 2, N-acetyl-β-D-mannosamine 1, fructose 1, citramalic acid, 5-dihydrocortisol 3 and 4-hydroxyphenylacetic acid were associated with periodontitis while, thymidine 3 and O-phosphoserine 1 were associated with health (**Pei 2020**). Additionally, uric acid, hypoxanthine, glutathione and ergothioneine all seem to be downregulated in periodontitis-affected sites (**Fornasaro 2021**).

A recent systematic review and meta-analysis on GCF-identified metabolites concluded that oxidative stress-related metabolites are mainly associated with periodontitis (malondialdehyde, 8-hydroxy-deoxyguanosine, 4-hydroxynonenal and neopterin) were metabolite that has been associated with periodontitis, while periodontal health was linked to glutathione (**Baima 2021**).

Few studies have assessed the sensitivity and specificity of biomarkers in GCF as diagnostic tests. A recent systematic review and meta-analysis, which presented the highest level of evidence identified in the literature, reported the MMP-8 is the GCF biomarker with the highest sensitivity (76.7%) and excellent specificity (92%) (**Arias-Bujanda 2019**). There is lack of metabolomic studies in the literature assessing sensitivity and specificity. This makes it the most consistent among the sum of GCF biomarkers to be possibly utilized in the diagnosis of periodontitis.

Studies on the Prognostic Value of Gingival Crevicular Fluid for Periodontitis

Over the years, multiple biomarkers have been identified in GCF as possible predictors of periodontitis' initiation and progression. In a comparison between patients that had periodontal disease progression and stable ones, Metalloproteinases 8 and 9 (MMP-8 & MMP-9), Osteoprotegerin (OPG) and Interleukin 1-β (IL-1β) were significantly higher in the periodontal disease progression group (**Kinney 2014**), and this was correlated with clinical measurements. MMP-8, which is in high concentration in patients with periodontitis has been shown to be one of the most reliable predictors of the periodontal disease onset and progression and may also be used as a monitoring agent in the periodontal maintenance periods, on a site-specific level (**Leppilahti 2015**).

It seems that, along with MMP-8, IL-1β is one of the most-commonly found biomarker found in the GCF of periodontitis patients as well as one of the most well-studied predicting factors about progression of periodontal disease (**Reinhardt 2010, Stadler 2016**). It has also been shown that their increase in periodontitis patients, compared to healthy individuals, correlates with an increase in the red and orange complex periodonto-pathogenic bacteria, as described by **Socransky** in **1998** (**Teles 2010)**. OPG, which is the

decoy receptor of RANKL, has, along with RANKL, also been detected in GCF and together they are known to play an important role in osteoclastic activity initiation and inhibition. Multiple other biomarkers have been identified in GCF as potential predictors of periodontitis initiation and progression with much less literature to support them.

More definitive results were found with alkaline phosphatase, which has been identified as a biochemical marker with application in detection and progression of periodontitis (**Sanikop 2012**).

Concluding from the available studies in the literature, identified metabolites in the GCF may be used as diagnostic tools for periodontal disease; even more they could be used in a site-specific manner. However, there is total lack of longitudinal metabolomic studies in the literature, investigating the value of metabolites as predictors of periodontal disease.

Is there a way to use the metabolites that can be analyzed from the GCF to make a diagnostic and predictive test for periodontitis that is accurate and painless? It is the objective of this study to generate a prediction model capable of assigning any new GCF sample to the category of health or disease. Another aim of this project is to provide the tools to accurately predict disease initiation and disease progression before it shows clinical and radiographic signs in a site-specific manner as well as to identify the specific metabolic pathways that these metabolites are part of (pre-cursors and post-metabolites).

We hypothesize that metabolites in gingival crevicular fluid through NMR and MS will predict future periodontal loss and distinguish between healthy and diseased sites.

II. Original Article

Metabolites in gingival crevicular fluid identified through Mass Spectrometry to diagnose periodontitis. A pilot study

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Abstract

Background: The technological advances in the field of metabolomics have shown promise for the development of a site-specific diagnostic test for periodontitis using the gingival crevicular fluid (GCF). We hypothesize that metabolites, identified through Mass Spectrometry (MS) will distinguish between health and disease.

Methods: Ten stage III/IV periodontitis patients were recruited and ten healthy controls and GCF samples from the 6 Ramfjord teeth and 2 randomly selected sites were harvested from subjects with a porous silver disc. The samples were analyzed with Hydrophilic interaction liquid chromatography (HILIC) and reverse-phase liquid chromatography (RPLC) coupled with tandem MS. Statistical analysis was performed using mixed linear models and the p-value was adjusted to control false discovery rate (FDR) using the method of Benjamini and Hochberg. Metaboanalyst 5.0 (www.metaboanalyst.ca) was utilized for pathway analysis.

Results: 3,491 metabolites were identified from the sample analysis and 108 could be matched to existing libraries. Those metabolites can be statistically assigned ($p<0.05$) to 12 human metabolic pathways. Butyric acid was associated healthy sites of healthy subjects, while Thymine, L-Theanine, Cytokinin B, L-Aspartic acid, D-Galactose, Citruline and Stachyose were associated with diseased sites of diseased subjects with an FDR <5%. The remaining identified compounds presented with a higher FDR. More compounds were detected with FDR <5%, but could not be identified due to warranting a targeted approach.

Conclusion(s): GCF metabolites identified through MS could be a potential future means to diagnose periodontitis. Butyric acid was associated with periodontal health and thymine, L-theanine, Cytokinin B, L-aspartic acid, D-galactose, citrulline and stachyose were associated with periodontitis. Longitudinal comparisons and a larger sample could show value in the development of a predictive test/model as well.

1. INTRODUCTION

Periodontitis is characterized by microbially-associated, host-mediated inflammation that results in loss of periodontal attachment and its diagnosis is currently based on the use of the periodontal probe (**Tonetti et. al. 2018**). This instrument is utilized to conduct measurements in the dento-gingival sulcus in millimeters (mm). This is a rather rudimentary tool, which is uncomfortable for the patient and identifies periodontitis after it has developed. It also entails measurement error (±1mm) by the examiner (**Watts 1995**). More contemporary tests that measure the periodontal microorganisms, proteins and enzymes in the periodontal pocket have low sensitivity and specificity to detect the progression or initiation of periodontal disease (**Armitage 2003**). Currently, there is no accurate way to predict the initiation and progression of periodontal disease. This is inefficient and expensive as an approach and it leads to unnecessary treatment for multiple patients. In 2015, the greatest losses in productivity were associated with tooth loss ($126.67 billion – 67%) and severe periodontitis ($38.85 billion – 21%) (**Righolt 2018**).

In an effort to create the specific diagnostic and prognostic test for periodontitis, oral fluids have been analyzed for potential biomarkers. The gingival crevicular fluid (GCF) is a physiological fluid and an inflammatory exudate which becomes inflammatory exudate in the presence of disease; thus, it has been used as a diagnostic aid since the 1970s (**Golub 1976, Smith 1977, Barros et. al 2016**).

The value of microbiological testing for periodontitis the different types of periodontal diseases is a subject of controversy and there is not a single one that has ideal characteristics (**Sanz 2004**). The "-omics" field shows promise in the development of an appropriate test

for the diagnosis, progression detection of periodontitis, as well as to determine the differential effects of treatment (**Callif 2017**).

The genomic investigations identified few loci, mainly for aggressive and severe forms of the periodontitis, with most studies having small or moderate sample sizes and great heterogeneity (**Morelli 2020**). Usually, genomic and also transcriptomic studies have been focusing on one or limited candidate genes/transcripts. In these studies, classic inflammatory mediators have been identified (**Trindale 2014**). Proteomics is able to evaluate the complete protein and peptide in health and periodontitis. Proteomics has not yet given definitive answers in our understanding of the pathogenesis of periodontal disease, other than multiple, rather than single, proteins have to be considered for characterizing the disease efficiently (**Bostanci 2018**). In the 1990s, Smith is the one that starts to look into GCF proteomics in a site-specific way (**Smith 1991, 1992**).

The most recent studies, however, focus on metabolomics, which has not yet been studied in equal detail to the previous three branches of the multi-omics (**Veensra 2012**). If we go back to the simplistic model of gene, transcript, protein and metabolite, our greatest chance in finding truly useful disease biomarkers will be studies that show correlation between biomolecules at all four levels. Metabolomics has gained popularity since it profiles directly the phenotype and changes thereof in contrast to other "-omics" technologies (**Amberg 2017**). Metabolomics' ultimate aim is to obtain a complete screen of all the metabolites of a given biological sample and interpret how the metabolic profile changes with a given pathophysiological state.

However, the technology to study metabolomics only recently reached its peak. The main techniques to study metabolomics are Mass Spectrometry (MS) and Nuclear Magnetic

Resonance (NMR) spectroscopy. NMR and MS both have their own advantages and disadvantages in conduction of metabolomic studies. MS's greatest advantage is its high sensitivity, with the ability to detect numerous species in just one sample (**Veenstra 2012**, **Emwas 2015**). While, NMR is precise and unbiased, it has low sensitivity and cannot detect nearly as many compounds as MS does.

While there are more studies on other fluids, such as saliva, serum and even studies on plaque, there are not enough studies on GCF yet (**Takahashi 2010, Elabdeen 2013, Sakanaka 2017, Chen 2018**).

A recent systematic review investigating the diagnostic value of saliva samples metabolomics concluded that valine, phenylalanine, isoleucine, tyrosine and butyrate are increased in periodontitis-affected subjects, whereas lactate, pyruvate and N-acetyl groups were increased in periodontally healthy patients (**Baima 2021**). Additionally, salivary samples-detected metabolites cadaverine, 5-oxoproline, and histidine are associated with periodontitis and could potentially predict the onset and progression of that disease (**Kuboniwa 2016**).

Whereas, saliva is more readily available compared to GCF, the latter has the advantage that it can reveal data on the metabolic profile on periodontitis in a site-specific way. A recent systematic review and meta-analysis on metabolites identified from GCF, came to the conclusion that oxidative stress-related metabolites are mainly associated with periodontitis (malondialdehyde, 8-hydroxy-deoxyguanosine, 4-hydroxynonenal and neopterin) were linked to periodontitis, whereas glutathione to periodontal health (**Baima 2021**). There is total lack of metabolomic studies on the prognostic value GCF-identified metabolites.

It is the aim of this study to generate a model capable of assigning any new GCF sample to the category of health or disease, in a site-specific manner. Additionally, to identify the specific metabolic pathways that these metabolites are part of (pre-cursors and post-metabolites). We hypothesize that metabolites in gingival crevicular fluid identified through MS will distinguish between healthy and diseased sites.

2. MATERIALS AND METHODS

2.1 Study Design and Population

Subjects with periodontitis and healthy controls that participated in this study were recruited at the University of Minnesota School of Dentistry in the time period of October 2018 to January 2020. Upon performing a periodontal examination and screening visit, eligibility for the study was confirmed according to the inclusion and exclusion criteria. Written consent form and Health Insurance Portability and Accountability Act (HIPPA) forms were subsequently signed by the patient prior to the beginning of the study under University of Minnesota Institutional Review Board (IRB) protocol #1511M79922.

Ten periodontally healthy subjects and 10 subjects with periodontitis were selected and recruited based on the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. The inclusion and exclusion criteria for the “test” and “control” group of this study are presented in Table 1 (**Chapple 2017, Papapanou 2018**).

TABLE 1 Description of inclusion and exclusion criteria of the periodontitis (test) & healthy (control) groups

Inclusion Criteria	**Exclusion Criteria**
Periodontitis (Test) Group	
Stage III or IV/Generalized Severe Periodontitis[1]	uncontrolled systemic diseases
at least 5 teeth with CAL ≥ 5 mm	systemic antibiotics within the last 3 months
at least 1 site with PD ≥ 7 mm	history of periodontal treatment or local antibiotics within the last 12 months
≥ 33% of radiographic bone loss	
Healthy (Control) Group	
PD ≤ 3 mm	uncontrolled systemic diseases
CAL = 0	systemic antibiotics within the last 3 months
no evidence of radiographic bone loss	PD ≥ 4 mm
	CAL ≥ 1 mm
	evidence of radiographic bone loss

[1] Periodontitis classifications based of 2017 Workshop for a new classification of Periodontal and Peri-implant Diseases and Conditions (**Papapanou 2018**) classification

2.2 Clinical Examination and Sampling Technique

All participants of the study were screened by residents or faculty of the Graduate Periodontology Clinic (Department of Developmental and Surgical Sciences) at the University of Minnesota. All involved investigators (M.C. – Primary Investigator, I.K. – Co-Investigator) underwent intra- and inter-examiner calibration for the measurement of PD and CAL.

Every patient had full-mouth CAL and PD charted at the beginning of the study, with full-mouth plaque, gingival bleeding and gingival index scores performed at all 6 sites (mesio-buccal, straight-buccal, disto-buccal, mesio-lingual, straight-lingual, disto-lingual) of

existing teeth as a part of the screening process (**O'Leary 1972, Newbrun 1996**). After patient selection, according to inclusion and exclusion criteria, GCF samples were collected between 2 and 8 weeks from the screening visit. Six Ramfjörd teeth plus two additional teeth with the deepest pockets in the mouth were sampled for a total of 8 sites per patient (**Ramfjord 1967**). At the sampling visit, proper isolation from salivary contamination was secured with a cotton roll or gauze. Supragingival plaque was carefully removed with a curette, to avoid bleeding from the gingival margin or pushing supragingival plaque in the pocket. The sulcus entrance was dried using a light stream of air from an air-water syringe directed away from the sulcus and towards the coronal aspect of the tooth. A 50 μm thin porous (5 μm pores) silver disk 3.96 mm in diameter (Sterlitech [cat#AG5048]) was slid into the healthy gingival sulcus with cotton pliers until light resistance was felt for a total of 30 seconds. Samples were discarded if visibly contaminated with blood, plaque or saliva. Saliva from the dorsal surface of the tongue was also sampled. Subjects were asked to swallow excess saliva before placing the membrane on the dorsal side of the tongue for 30 seconds. Two negative control samples, one with elution buffer alone and the other with an untouched silver disk plus buffer were also collected. Experimental and control samples were numbered sequentially throughout the study for experimental rigor to connect clinical measurements and metabolomic data for each samples site. Measurements were conducted using a Michigan probe with Williams markings (1-2-3-5-7-8-9-10 mm) (Hu-Friedy, IL, USA) with light to moderate pressure into the sulcus. PD was measured from the free gingival margin to the base of the pocket and CAL measurements were taken from the CEJ to the base of pocket or from a restorative margin to the base of pocket if the CEJ was not present. All sampled sites were checked

for presence or absence of plaque by sliding the probe supra-gingivally at the selected site. Bleeding on probing was assessed 30 seconds after probing and noted as profuse, pin-point or absent. Full-mouth measurements were taken as described in the screening visit and were collected again at the baseline visit only from the eight selected sites to obtain a "double set" of measurements. Full-mouth peri-apical radiographs were taken at the beginning of the study and vertical bitewings were taken at the initial visit, using film holders with long cone paralleling, with phosphor plates or sensors. History of periodontal disease and new disease or extent of disease progression were measured as CAL change at each site.

2.3 GCF sample processing

At the sampling visit the silver disks were placed in an Eppendorf tube (TUBE A) and stored dry and frozen at -80 C within 4 hours. At elution day, each silver disk was thawed, and 100 μL of Mass Spectrometry Grade water was added to each Eppendorf tube. Samples were vortexed for 30 seconds and centrifuged at 13,200 rpm (Microfuge-Eppendorf) for 1 minute. Then the 100 μL solution was split into a 60 μL aliquot to submit for MS (TUBE B) and a 40 μL aliquot for NMR (TUBE C); TUBE C was stored as a part of an ongoing longitudinal investigation that includes comparison of MS with NMR. To the NMR sample aliquot, we added 10 μL of D20 (deuterium water) + 2,2-Dimethyl-2-silapentane-5-sulfonic acid (DSS) [5x concentrated] + PBS [5x concentrated] + AZIDE [5x concentrated]. Following that, tubes B and C were frozen again at -80 C.

Twenty-four hours prior to NMR analysis day, Tube C was thawed and immediately loaded in a borosilicate tube 1.7mm X 103.5 mm (SampleJet [cat #Z106462] – Bruker - Germany)

with long gel-loading pipet tips (Sorenson [Cat#: 13810]) and stored at +4°C. All samples were stored in a ~5°C refrigerator or cooled SampleJet sample changer at ~5°C while awaiting NMR acquisition.

The thawed Eppendorf tube (TUBE B) was delivered to the Center of Mass Spectrometry and Proteomics (CMSP) lab in dry ice. The developed protocols of CMSP for processing of the sample followed the standards described in the current literature based on other biologic fluids (**Bernini 2011**, **Dunn 2011, Dunn 2012, Broadhurst 2018**). The protocol included: addition of heavy-atom (deuterated or 13C) and 4 volumes (400 µl) of chilled (-20°C) 90/10 methanol/acetone to each sample. The samples were then vortexed at high speed for 1 min, incubated at -20°C for 15 min, centrifuged at 13,000 x g, 15 min, 4°C and transferred to new tubes. Subsequently, the samples were evaporated to dryness under a stream of inert (nitrogen) gas and reconstituted in 5% acetonitrile, 0.1% formic acid, vortexed and centrifuged again (13,000 x g, 5 min, 4°C). The supernatants were then transferred to MS vials containing the indicated volume of 10% formic acid and stored at -70°C or -80°C until used for mass spectrometry.

Hydrophilic interaction liquid chromatography (HILIC) was used which offers additional information to reverse-phase liquid chromatography (RPLC) by retaining polar metabolites (**Contrepois 2015**). HILIC is based on the hydrophilicity of the compound; non-polar compounds are detected first, followed by polar ones. On the contrary, RPLC is based on the hydrophobicity of the compound, with the most polar compounds being detected first, followed by the non-polar ones. Thus, with the combination of the two methods, the maximum number of compounds can be detected. RPLC was run in a "positive" (observed ion has received a H^+) and HILIC was run in a "negative" (observed ion has lost a H^+) ion

mode. Available software – MZMine2 (**Pluskal 2010**) and XCMS (**Smith 2006**) – was used to process the MS-derived signal in order to achieve metabolite profiling. The metabolites were matched to available libraries: HMDB (**Wishart 2008**), the KEGG (**Okuda 2008**), NIST17 (**Stein 2012**) and MassBank (**Horai 2010**). The identified metabolites were submitted for pathway analysis to MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/) (**Chong 2019**).

2.4 Statistical Analysis

Prior to analysis, metabolite measurements (relative abundance) were normalized to total sample quantity by dividing each measurement by the total of all measurements in that sample. Next, each metabolite was log-transformed to have an approximately normal distribution, then centered and scaled to have mean 0 and standard deviation 1. Each metabolite was then compared between the three groups (H/H, D/D, H/D) using mixed-effects linear models, with a fixed effect for group and a random effect for participant to account for dependence among sites in the same participant. To account for multiple comparisons of metabolites, p-values for these comparisons were adjusted, using the method of Benjamini and Hochberg (**Benjamini 2015**), to control the false discovery rate (FDR). FDR is the expected proportion of false positive findings among all positive findings. Clustered image maps (heat maps) were used to visualize patterns among metabolite levels, sites, and groups, with metabolites and sites each ordered using hierarchical clustering to place similar metabolites or sites in close proximity. PLS-DA was used to construct and examine the feasibility of a prediction model with group as the outcome and metabolites as predictors. Analyses were conducted using R version 4.0.3 (**R**

Core Team 2020) including the packages lme4 version 1.1-26 (**Bates 2015**) and mixOmics version 6.12.2 (**Rohart 2017**).

3. RESULTS

A total of 20 subjects were recruited to the study. Of these subjects, 10 are test and 10 are control subjects. In order to determine whether the membrane should be frozen dry or in PBS, a preliminary run was done. From the original pool of subjects, 4 samples were selected to be analyzed by Mass Spectrometry. 2 of these samples were stored dry, according to the above-described protocol and 2 were immersed in 60 μl of PBS and then frozen. From these samples, 14,801 metabolites were identified with Hydrophilic Interaction Liquid Chromatography (HILIC) coupled with tandem Mass Spectrometry. 2,677 were matched to existing libraries and analyzed. 1,719 metabolites were better eluted from dry samples and 958 from wet samples, making elution from dry samples more suitable for analysis.

12 metabolic pathways were found to be significantly associated with PD ≥4 mm (**Fig. 1**). The aminoacyl-tRNA biosynthesis pathway was associated with the greatest significance. The phenylalanine, tyrosine & tryptophan as well as the taurine and hypotaurine pathway had the greatest pathway impact when PD ≥4 mm; the increased pathway impact is associated with the number of metabolites which is observed in a specific metabolic pathway. Other pathways that were considered significant ($P<0.05$) were the following: arginine biosynthesis; alanine, aspartate and glutamate metabolism; D-Glutamine and D-glutamate metabolism; Citrate cycle (TCA cycle); butanoate

metabolism; galactose metabolism; nitrogen metabolism; glyoxylate and dicarboxylate metabolism; histidine metabolism. The stratification of the samples in shallower [4-6 mm] and deeper [≥7 mm] pockets revealed the same significant pathways, but the level of significance was greater in the deeper pockets compared to the shallower ones.

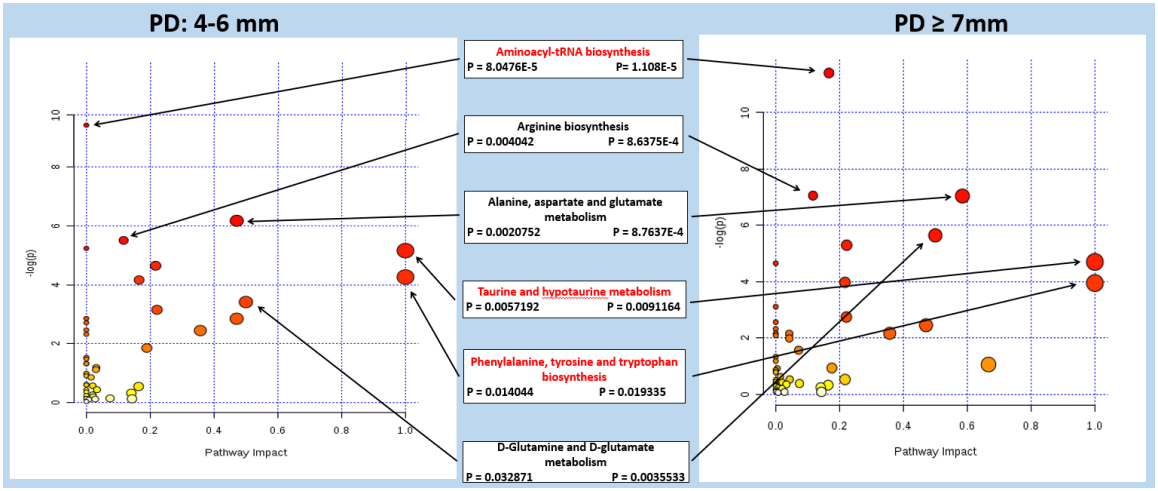


FIGURE 1 All matched significant pathways ($p<0.05$) from the pilot run are represented by circles, with the color of each circle corresponding to its p-value (y axis) and the size of the circle corresponding to its pathway impact value (x axis). The pathway impact depends on the number of metabolites that are seen in a specific metabolic pathway.

Following the preliminary run, 220 samples were selected. 10 of which belonged to test individuals and 10 from healthy controls. All samples were randomized to assure that the individual conducting the MS processing is blind to which samples belongs to the test or control group. 402 compounds were detected with HILIC and 3,089 compounds were detected RPLC, indicating an expected superiority of RPLC in detecting a larger number of metabolites. Of the compounds identified with HILIC, only 42 metabolites could be initially identified and matched to existing libraries. Similarly, of all the compounds detected with RPLC only 66 metabolites were identified and matched.

The metabolites identified by both RPLC and HILIC combined can be statistically assigned ($p<0.05$) to 12 human metabolic pathways (**Fig. 2**): 1. Aminoacyl-t-RNA biosynthesis; 2. arginine biosynthesis; 3. valine, leucine and isoleucine biosynthesis; 4. purine metabolism; 5. glycine, serine and threonine metabolism; 6. D-glutamine and D-glutamate metabolism; 7. phenylalanine, tyrosine and tryptophan biosynthesis; 8. arginine and proline metabolism; 9. butanoate metabolism; 10. histidine metabolism; 11. alanine aspartate and glutamate metabolism; 12. pantothenate and CoA biosynthesis. The remaining 3,383 compounds remained un-matched, warranting a "targeted" approach to identify them.

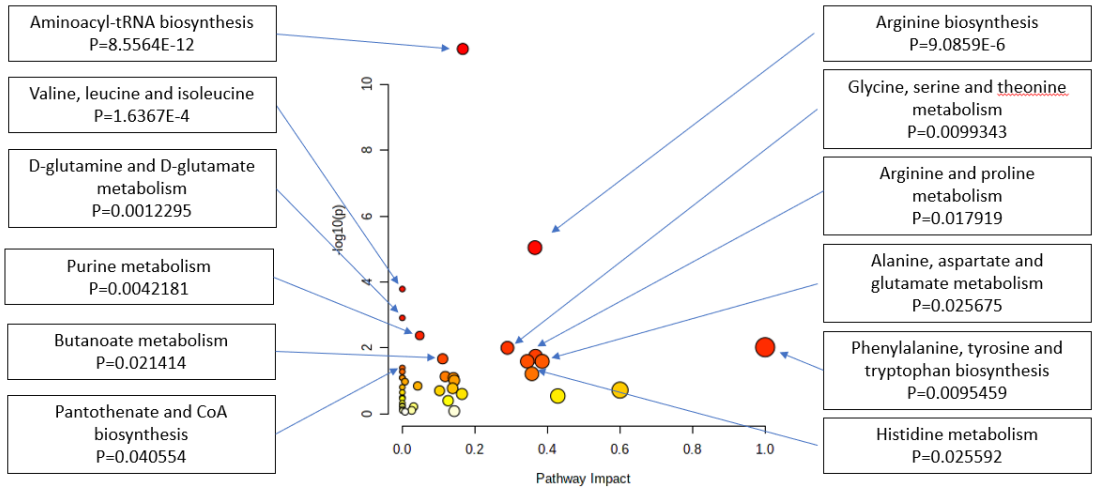


FIGURE 2 All matched significant pathways ($p<0.05$) from the untargeted RPLC and HILIC coupled with tandem MS are represented by circles, with the color of each circle corresponding to its p-value (y axis) and the size of the circle corresponding to its pathway impact value (x axis). The pathway impact depends on the number of metabolites that are seen in a specific metabolic pathway.

From the untargeted approach of the positive run of MS volcano plots were made to represent comparisons among the groups of sites (**Fig. 3**). In the volcano plots each compound, identified and unidentified, is represented by a single, colored circle. The x axis represents the mean difference (the absolute difference between the mean value in the two compared groups) and the y-axis represents the FDR. A metabolite that is located far from 0 on the x-axis has a greater difference in the means of the compared groups, while the +/- represents the group that the metabolite is associated with. The color of the circle represents the false discovery rate (FDR). The FDR is calculated based on adjusting the p-value adjusted using the method of Benjamini and Hochberg. The circles are large and colored pink, if FDR is <5% or significantly correlated with disease or with health, green and medium-sized if FDR=5-20%. The small and yellow circles represent an FDR >20%. The identified metabolites have their names written by their assigned circle. As seen, the majority of circles with FDR <5% were unidentified, indicating the necessity of a targeted approach.

When metabolites in "healthy sites of healthy subjects" (H/H), were compared to metabolites in "diseased sites of diseased subjects" (D/D) (**Fig. 3A**) it appears that all the metabolites with a low FDR were not identified. However, Guanosine, Raffinose, Creatine, N-Nonanoylglycine and N-Deconoylglycine were positively associated with H/H, while Glyoylproline was positively associated with D/D with an FDR=5-20%.

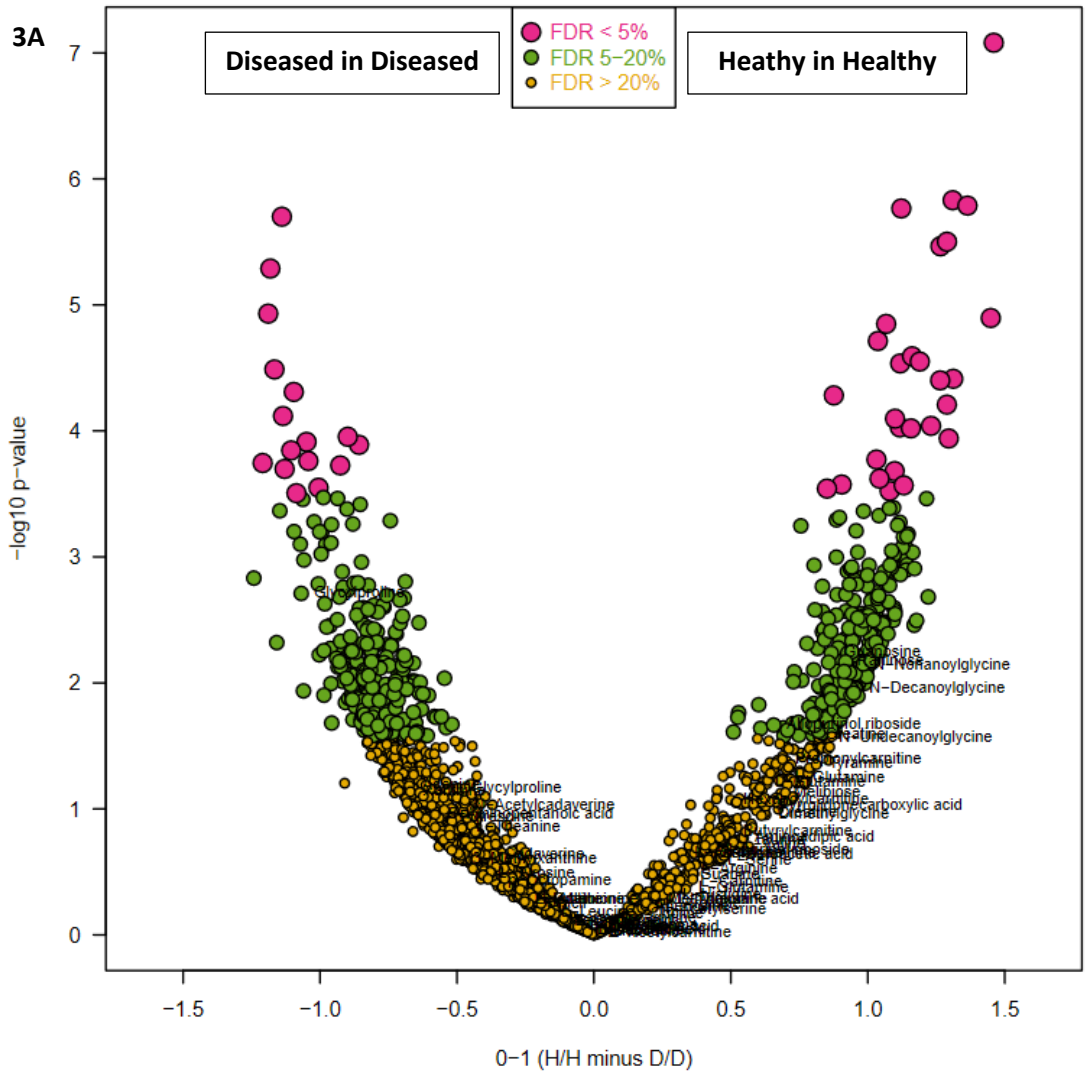
When metabolites H/H were compared to metabolites in "healthy sites of diseased subjects" (H/D) the metabolites Allopurinol riboside, N-Nonanoylglycine, Raffinose, Creatine, Pripionylcarnitine, L-Glutamine and Guanosine were positively associated with

H/H. Metabolites (**Fig. 3B**). No metabolites positively associated with H/D with FDR<20% were identified, and for both groups, all the metabolites with FDR<5%

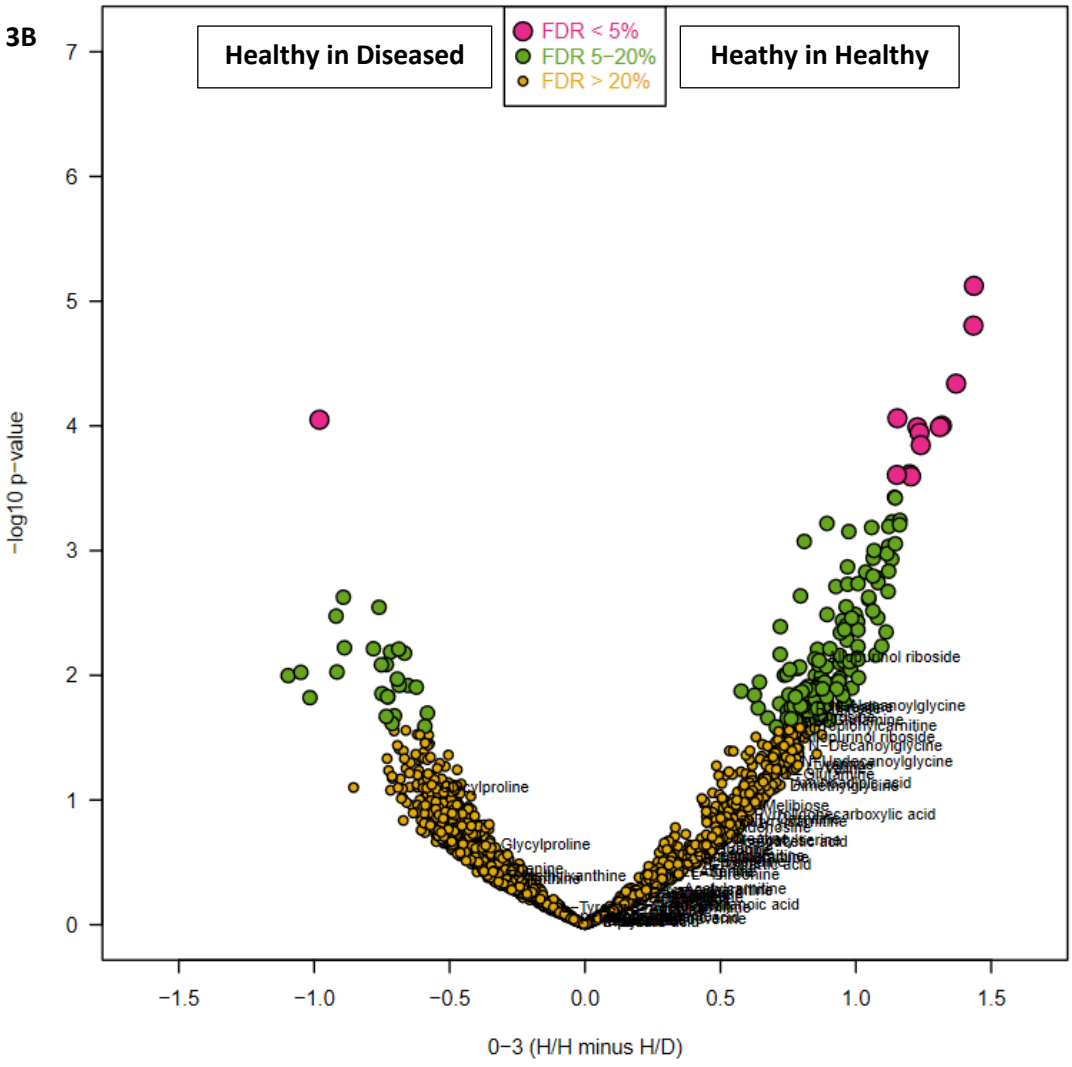
remained unidentified, warranting a targeted approach.

When metabolites in D/D were compared to metabolites in H/D there were only 2 detected metabolites positively associated with disease with an FDR<5%, both unidentified (**Fig. 3C**). 5-Aminopentanoic acid, L-Proline, L-Aspartic acid, Putrescine, Glycosylproline, L-Theanine, L-Carnitine and Cadaverine were positively associated with D/D with an FDR=5-20%.

3A


Diseased in Diseased
FDR < 5%
FDR 5−20%
FDR > 20%
Heathy in Healthy
−log10 p−value
0−1 (H/H minus D/D)
7
6
5
4
3
2
1
0
−1.5
−1.0
−0.5
0.0
0.5
1.0
1.5
N−Decanoylglycine
N−Undecanoylglycine
Acetylcadaverine
Glycylproline
Hexanoylcarnitine
Octanoylcarnitine
Decanoylcarnitine
Dodecanoylcarnitine
Tetradecanoylcarnitine
Hexadecanoylcarnitine
Octadecanoylcarnitine

3B


Healthy in Diseased
FDR < 5%
FDR 5−20%
FDR > 20%
Heathy in Healthy
−log10 p−value
0−3 (H/H minus H/D)
Glycylproline
Decanoylglycine
Melibiose

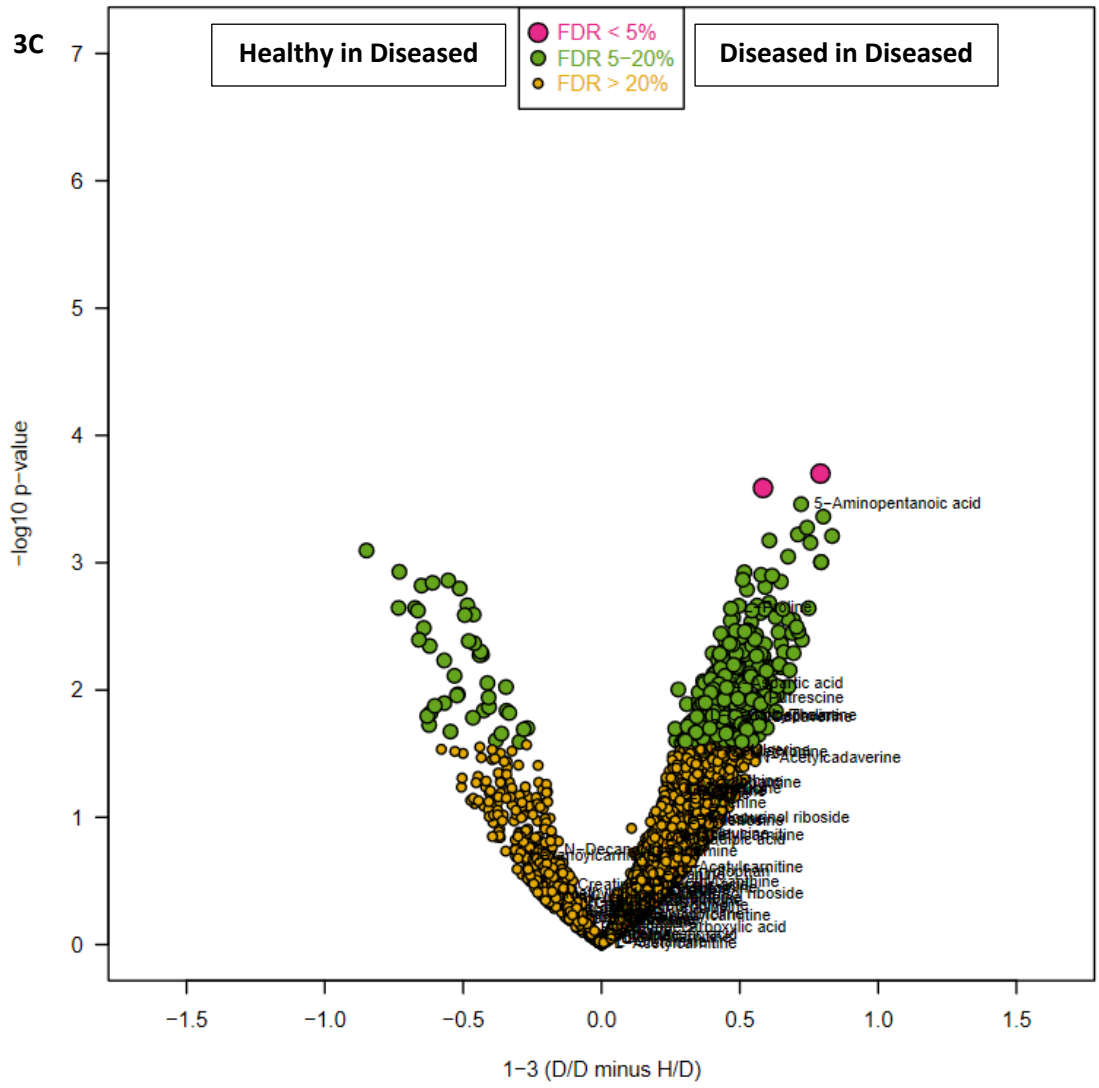


FIGURE 3. Volcano plots representing comparisons among predetermined groups having occurred from the RPLC-MS (untargeted). **3A** Comparison of H/H vs. D/D. **3B** Comparison of H/H vs. H/D. **3C** Comparison of D/D vs. H/D

In the volcano plots each compound, identified and unidentified, is represented by a single, colored circle. The circles are colored pink, if the false discovery rate (FDR) is <5%, green if FDR=5-20% and yellow if FDR >20%. The identified metabolites have their names written by their assigned circle. Most circles with FDR <5% were not identified. This indicates the need for a targeting approach.

(H/H): healthy sites in healthy subjects; (D/D): diseased sites in diseased subjects;

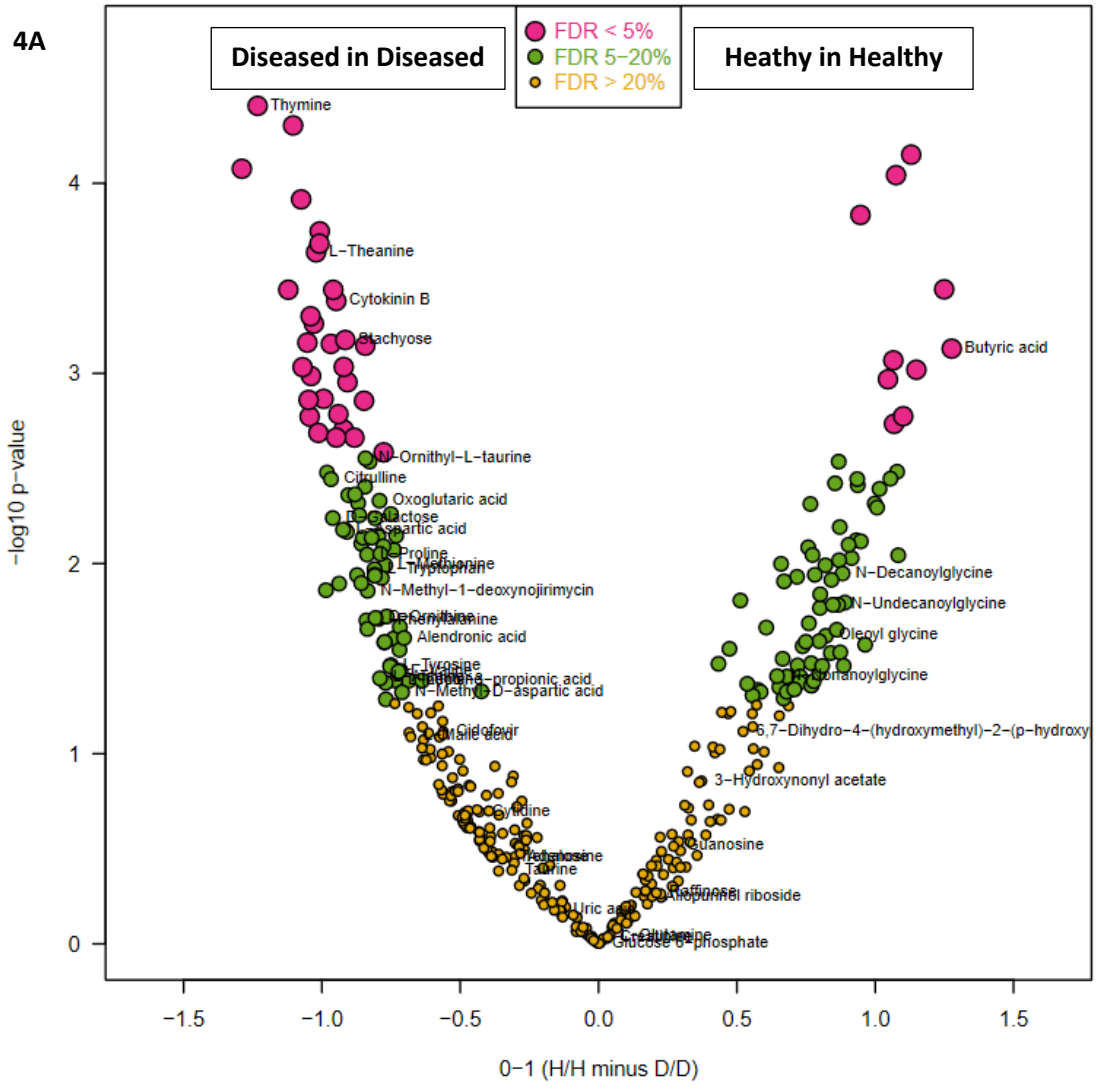
(H/D): healthy sites in diseased subjects

Similarly, HILIC-MS, volcano plots were made to represent comparisons among the groups of sites (**Fig. 4**). When metabolites in H/H, were compared to metabolites in D/D (**Fig. 4A**), Butyric acid was positively associated H/H and also Thymine, L-Theanine, Cytokinin B and Stachyose were positively associated with D/D with an FDR<5%. Expanding to an FDR=5-20%, N-Ortnithine-L-Taurine, Citrulline, Oxoglutaric acid, D-Galactose, L-Aspartic acid, L-Proline, L-Methionine, L-Tryptophan, N-Methyl-1-deoxynojirimycin, D-Ornithine, L-Phenylalanine, Alendronic acid, L-Tyrosine, L-Valine, L-Alanine, L-Leucine, Indole-3-propionic acid and N-Methyl-D-aspartic acid had positive associations with D/D. N-Decanoylglycine, N-Undecanoylglycine, N-Nonanoylglycine and Oleoyl glycine were positively associated with H/H with FDR=5-20%,

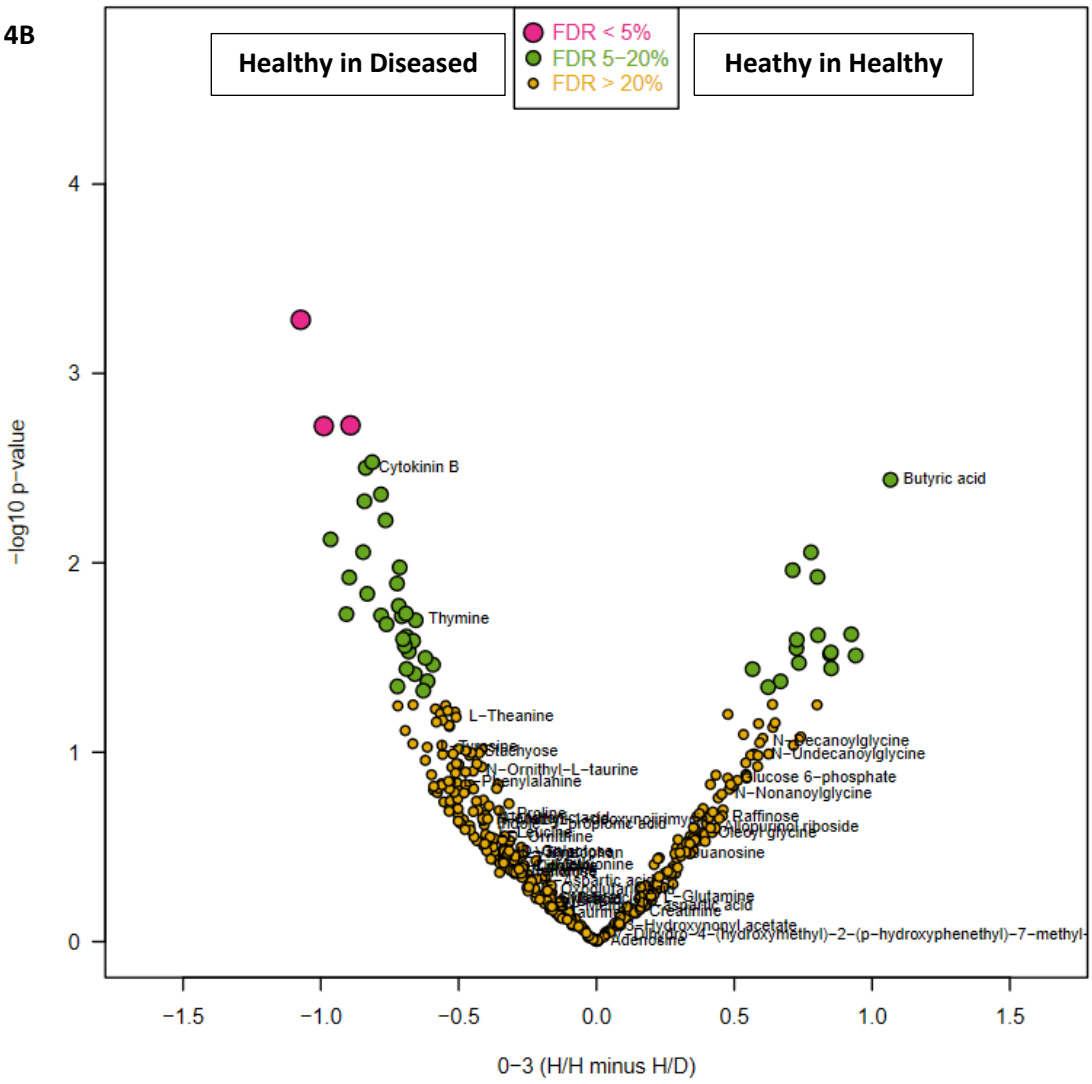
When metabolites in H/H were compared to metabolites in H/D, the metabolite Butyric acid was positively associated with H/H, whereas Thymine and Cytokinin B were positively associated with H/D with an FDR=5-20% (**Fig. 4B**). This latter result indicates that H/D sites are more similar to D/D sites than to H/H sites. Only 3 metabolites were detected with an FDR<5% for the group H/D and they all remained unidentified.

When metabolites in D/D were compared to metabolites in H/D, L-Aspartic acid, D-Galactose and Citruline were positively associated with D/D with an FDR<5% (**Fig. 4C**). Positively associated with D/D, with FDR=5-20% were: Thymine, N-Methy-D-Aspartic acid, Thymine, L-Theanine, Oxoglutaric acid, L-Methionine, L-Tryptophan, N-Methyl-1-deoxynojirimycin, D-Ornithine, L-Alanine, N-Methyl-D-aspartic acid and L-Malic acid. Finally, Oleoylglycine and 6,7-Dihydro-4-(hydroxymethyl)-2-(p-hydroxyphenethyl)-7-methyl-5H-2-pyrindinium were positively associated with H/D with an FDR=5-20%.

4A


Diseased in Diseased
Heathy in Healthy
FDR < 5%
FDR 5−20%
FDR > 20%
Thymine
L−Theanine
Cytokinin B
Stachyose
Butyric acid
N−Ornithyl−L−taurine
Citrulline
Oxoglutaric acid
Proline
N−Methyl−1−deoxynojirimycin
Alendronic acid
N−Decanoylglycine
N−Undecanoylglycine
Oleoyl glycine
3−Hydroxynonyl acetate
Guanosine
Raffinose
Glucose 6−phosphate
−log10 p−value
0−1 (H/H minus D/D)
4
3
2
1
0
−1.5
−1.0
−0.5
0.0
0.5
1.0
1.5

4B


Healthy in Diseased
FDR < 5%
FDR 5−20%
FDR > 20%
Heathy in Healthy
−log10 p−value
0−3 (H/H minus H/D)
Cytokinin B
Butyric acid
Thymine
L−Theanine
Stachyose
N−Ornithyl−L−taurine
Phenylalanine
Glucose 6−phosphate
N−Nonanoylglycine
Raffinose
Oleoyl glycine
Guanosine
L−Glutamine
Creatinine
Adenosine

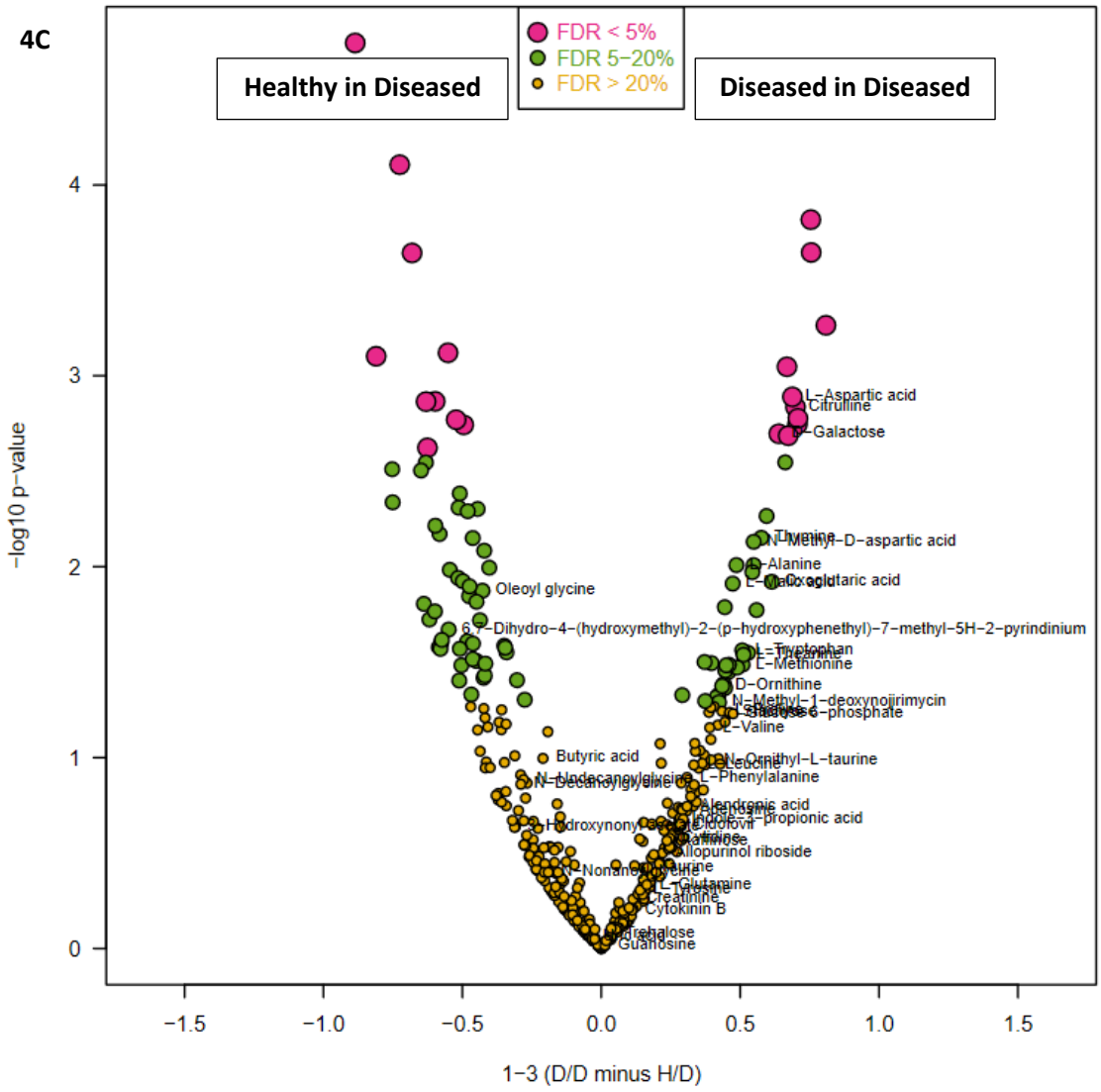


FIGURE 4. Volcano plots representing comparisons among predetermined groups having occurred from the HILIC-MS (untargeted). **4A** Comparison of H/H vs. D/D. **4B** Comparison of H/H vs. H/D. **4C** Comparison of D/D vs. H/D

In the volcano plots each compound, identified and unidentified, is represented by a single, colored circle. The circles are colored pink, if the false discovery rate (FDR) is <5%, green if FDR=5-20% and yellow if FDR >20%. The identified metabolites have their names written by their assigned circle. Most circles with FDR <5% were not identified. This indicates the need for a targeting approach.

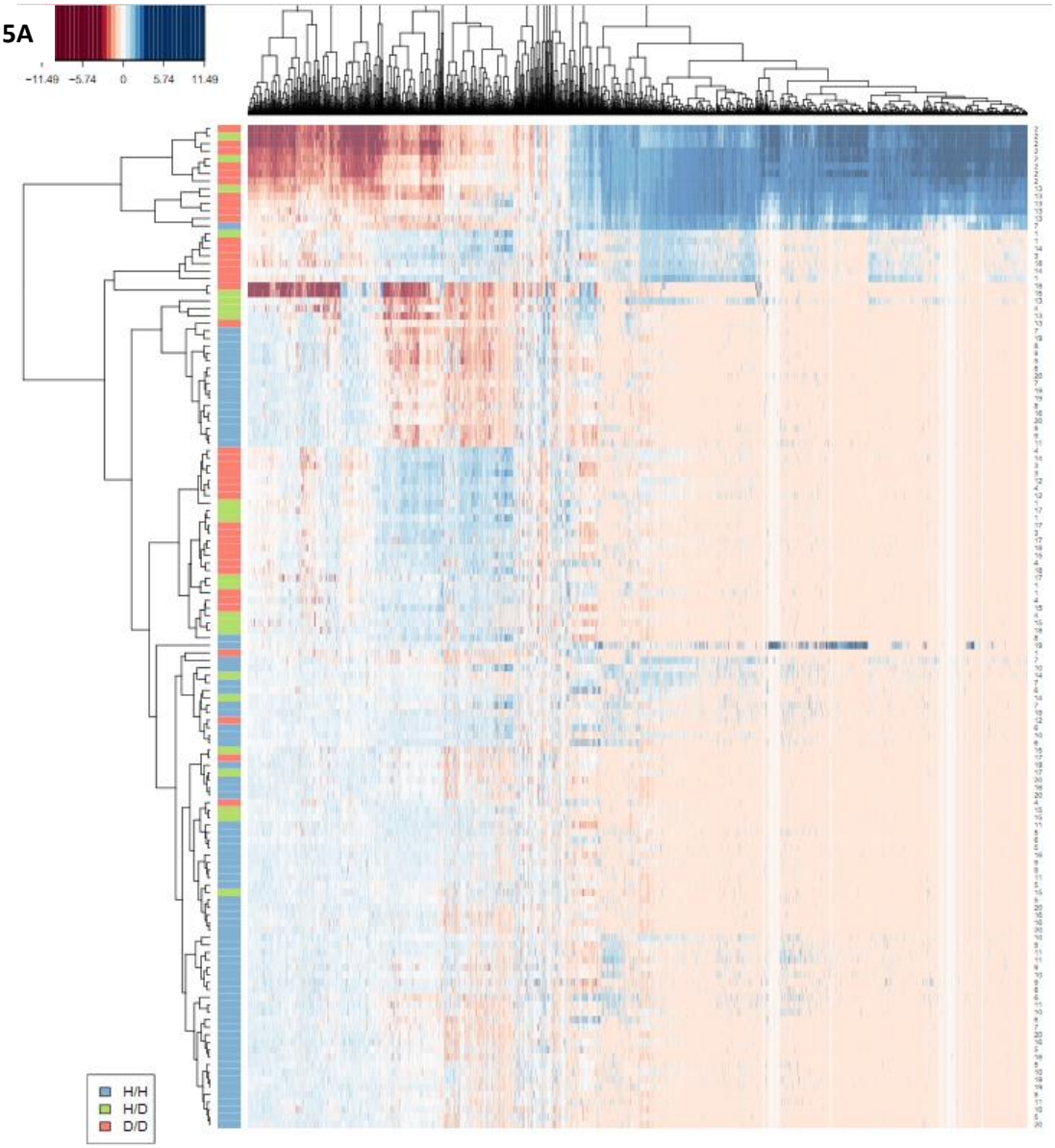
(H/H): healthy sites in healthy subjects; (D/D): diseased sites in diseased subjects;

(H/D): healthy sites in diseased subjects

A heatmap from the statistical analysis of the discovered metabolites grouped by subjects and sites, shows distinction from the top of the heatmap compared to the rest of it (**Fig. 5A**). In this heatmap, the y-axis is a dendrogram that shows clustering of similar sites grouped together. The x-axis dendrogram shows clustering of the metabolites that have similar associations with one another. The dark red color in the heatmap represents low levels of a metabolite and the dark blue high levels of a metabolite. As indicated in the right of the heatmap, these rows correspond to the sites sampled from two particular subjects (#2 and #13).

Similarly, from the PLS-DA analysis sites from subjects #2 and 13 appear quite differently from the ones from other subjects (**Fig. 5B**). The x-axis of the figure represents the single patient that samples derived from. The y-axis summarizes all the metabolites by a single score, with a higher score showing increased levels of the metabolites associated with disease and decreased levels of those metabolites associated with health.

5A



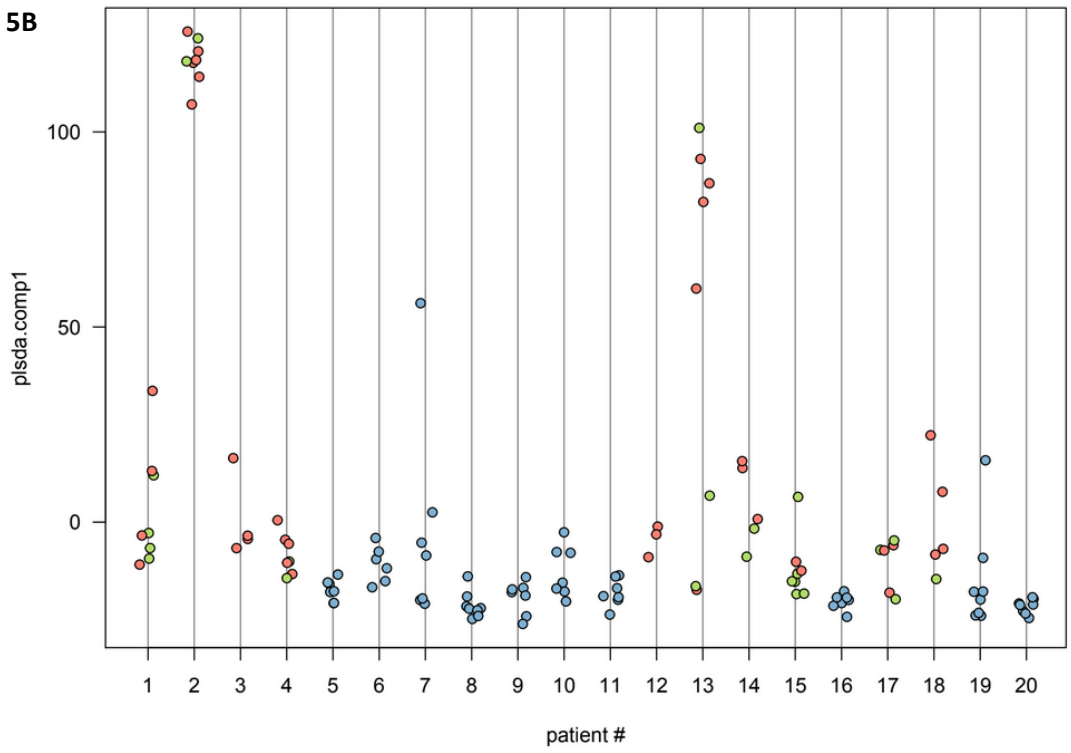


FIGURE 5. (**A)** Heatmap representing the detected compounds in sampled sites from the selected 20 subjects. In the heatmap. The red color represents low levels of a metabolite and the blue high levels of a metabolite. **(B)** PLS-DA: Representation of each patient and the individual sampled sites.
(H/H): healthy sites in healthy subjects (blue circle)
(D/D): diseased sites in diseased subjects (red circle)
(H/D): healthy sites in diseased subjects (green circle)

4. DISCUSSION

In a similarly designed, unpublished study by the same group, using just NMR the number of metabolites that were identified was 35. As expected, the much more sensitive MS detected a much larger number of metabolites. In order to incorporate the benefits of NMR to the quantitive benefits of MS, this study was only a "pilot" component of an ongoing investigation. This investigation will conduct a targeted MS analysis of the existing "pilot" analysis to increase the sample size to 40 test and 40 control subjects; longitudinal comparisons analyzing the samples by both MS and NMR will be made as well with a total follow-up of 2 years.

As the technologies of NMR and MS in metabolomics has just recently advanced, there still is a lack of studies in the literature. GCF can reveal the metabolic profile of periodontitis in a site-specific way. In the current literature, information on GCF metabolites associated with periodontitis is limited. In a study that compared GCF obtained from healthy, gingivitis and periodontitis subjects, the metabolic pathway of purine degradation, which is a major source for reactive oxygen species production, was significantly accelerated in periodontitis subjects (**Barnes 2009**). Metabolomic analysis through MS could be useful in prediction and diagnosis of the patient, showing distinct differences between healthy sites, shallower and deeper pockets, which is promising for the development of a new test (**Ozeki 2016**). Additionally, when extracting data from studies that utilized metabolomic analysis of both GCF and serum, more metabolites could be identified in the GCF (**Chen 2018**). In this study, both fluids could show significant differences between healthy and aggressive periodontitis subjects.

A recent study reported on the discovery of GCF metabolites associated with chronic periodontitis (**Pei 2020**). Uracil, N-carbamylglutamate 2, N-acetyl-β-D-mannosamine 1, fructose 1, citramalic acid, 5-dihydrocortisol 3 and 4-hydroxyphenylacetic acid. thymidine 3 and O-phosphoserine 1 were associated with health. Analysis of GCF from periodontitis subjects using surface enhanced Raman scattering has led to the identification of uric acid, hypoxanthine, glutathione and ergothioneine, all of which were reduced in cases of periodontitis (**Fornasaro 2021**).

A recent systematic review and meta-analysis from 15 studies on GCF metabolites, 11 of which were targeted, managed to identify a total of 10 metabolites with available information regarding their association with gingival health, gingivitis or periodontitis (**Baima 2021**). This study concluded that oxidative stress-related metabolites were primarily reported in association with periodontitis. Malondialdehyde, 8-hydroxy-deoxyguanosine, 4-hydroxynonenal and neopterin were metabolite positively associated with periodontitis. Conversely, glutathione was positively associated with periodontal health.

Salivary metabolites have also been studied as biomarkers for periodontitis. Cadaverine, 5-oxoproline, and histidine have been shown to be metabolites isolated in saliva that are positively associated with periodontitis (**Kuboniwa 2016**). Other identified salivary metabolites associated with periodontal disease are the dipeptides leucylisoleucine, phenylphenol, and serylisoleucine as well as the fatty acids arachidonate, arachidate, and dihomo-linolate are attractive candidate markers (**Barnes 2011**). The bacterial metabolite phenylacetate isolated from saliva has been significantly associated with periodontitis as well, making a potential biomarker for periodontitis (**Liebsch 2019**).

Tongue swabs and saline washouts have been also able to identify metabolites that are related to health and disease through NMR analysis (**Gawron 2019**). Additionally, the salivary metabolic fingerprints, through NMR, of chronic and aggressive periodontitis have been studied with no significant differences between the two conditions (**Romano 2018**). Furthermore, an NMR-based study showed that non-surgical periodontal treatment is able to lead to significant changes in the salivary metabolic profile of a periodontitis patient; however, it will still be distinct from the one of a healthy patient (**Romano 2019, Citterio 2020**). A recent systematic review on salivary metabolomics concluded that valine, phenylalanine, isoleucine, tyrosine and butyrate are increased in subjects with periodontitis (**Baima 2021**). Furthermore, that same study, found that lactate, pyruvate and N-acetyl groups were increased in periodontal health.

Returning to our results, and as it occurs from the statistical analysis of the untargeted approach with RPLC- and HILIC-MS, it is evident that both methods are valuable in detecting a large number of metabolites. The pilot analysis of these 20 subjects was valuable for many reasons. To begin with, a targeted approach can now be started to identify the metabolites that appear with an FDR <5%. This way, in future analyses of the samples to come, a targeted approach can be run from the start of the analysis. Secondly, it is established that the RPLC-MS provides a much larger number of metabolites compared to HILIC-MS. Additionally, there is a large number of metabolites that can clearly distinguish among the different groups as shown in Figures 5 and 6, thus suggesting that with the identification of these metabolites it is feasible to recognize a healthy site in a healthy patient, a diseased site in a diseased patient and a healthy site in a diseased patient from their metabolic "profile". Possibly of all the comparisons, the most important one is

the one between H/D and D/D, as of all the sites, the H/D are the most susceptible to become D/D in the future. It is interesting to follow-up the patients longitudinally to explore whether the changes in the metabolites correspond with disease progression. Finally, even from the limited number of metabolites identified with untargeted RPLC-MS and HILIC-MS (positive and negative mode), significant metabolic pathways could be identified (Figure 4). This is particularly promising considering that the targeted approach will identify a greater number of metabolites, significantly associated with health or disease, to be utilized for pathway analysis.

5. CONCLUSION

It appears that metabolites from the GCF of specific sites, identified through MS, are able to create a metabolic profile that can distinguish between health and disease. Butyric acid was positively associated with periodontal health whereas thymine, L-theanine, cytokinin B, L-aspartic acid, D-galactose, citrulline and stachyose were associated with periodontitis. This is promising because a diagnostic test can be developed in the future that can identify a healthy from a diseased site. Longitudinal comparisons pending, and with the recruitment of a larger sample of subjects, these data could also be of value in the development of a prognostic test as well. Additionally, it is promising, as suggested by the data acquired from this pilot analysis, to identify the specific metabolic pathways that these metabolites are part of (pre-cursors and post-metabolites).

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Amberg, A., B. Riefke, G. Schlotterbeck, A. Ross, H. Senn, F. Dieterle and M. Keck (2017). "NMR and MS Methods for Metabolomics." Methods Mol Biol 1641: 229-258.
2. Armitage, G. C., S. Research and P. Therapy Committee of the American Academy of (2003). "Diagnosis of periodontal diseases." J Periodontol 74(8): 1237-1247.
3. Baima G, Corana M, Iaderosa G, Romano F, Citterio F, Meoni G, Tenori L, Aimetti M. Metabolomics of gingival crevicular fluid to identify biomarkers for periodontitis: A systematic review with meta-analysis. J Periodontal Res. 2021 Mar 12. doi: 10.1111/jre.12872. Epub ahead of print. PMID: 33710624.
4. Baima G, Iaderosa G, Citterio F, Grossi S, Romano F, Berta GN, Buduneli N, Aimetti M. Salivary metabolomics for the diagnosis of periodontal diseases: a systematic review with methodological quality assessment. Metabolomics. 2021 Jan 1;17(1):1. doi: 10.1007/s11306-020-01754-3. PMID: 33387070.
5. Barnes VM, Teles R, Trivedi HM, Devizio W, Xu T, Mitchell MW, Milburn MV, Guo L. Acceleration of purine degradation by periodontal diseases. J Dent Res. 2009 Sep;88(9):851-5. doi: 10.1177/0022034509341967
6. Barnes, V. M., S. G. Ciancio, O. Shibly, T. Xu, W. Devizio, H. M. Trivedi, L. Guo and T. J. Jonsson (2011). "Metabolomics reveals elevated macromolecular degradation in periodontal disease." J Dent Res 90(11): 1293-1297.
7. Barros, S. P., R. Williams, S. Offenbacher and T. Morelli (2016). "Gingival crevicular fluid as a source of biomarkers for periodontitis." Periodontol 2000 70(1): 53-64.
8. Douglas Bates, Martin Maechler, Ben Bolker, Steve Walker (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48. doi:10.18637/jss.v067.i01
9. Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. _Journal of the Royal Statistical Society Series B_, *57*, 289-300.
10. Bernini P, Bertini I, Luchinat C, Nincheri P, Staderini S, Turano P. Standard operating procedures for pre-analytical handling of blood and urine for metabolomic studies and biobanks. J Biomol

NMR. 2011 Apr;49(3-4):231-43. doi: 10.1007/s10858-011-9489-1. Epub 2011 Mar 5. PubMed PMID: 21380509.

11. Bostanci, N. and G. N. Belibasakis (2018). "Gingival crevicular fluid and its immune mediators in the proteomic era." Periodontol 2000 76(1): 68-84.
12. Broadhurst D, Goodacre R, Reinke SN, Kuligowski J, Wilson ID, Lewis MR, Dunn WB. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. Metabolomics. 2018;14(6):72. doi: 10.1007/s11306-018-1367-3. Epub 2018 May 18. Review. PubMed PMID: 29805336; PubMed Central PMCID: PMC5960010.
13. Califf, K. J., K. Schwarzberg-Lipson, N. Garg, S. M. Gibbons, J. G. Caporaso, J. Slots, C. Cohen, P. C. Dorrestein and S. T. Kelley (2017). "Multi-omics Analysis of Periodontal Pocket Microbial Communities Pre- and Posttreatment." mSystems 2(3).
14. Chapple ILC, Mealey BL, Van Dyke TE, Bartold PM, Dommisch H, Eickholz P, Geisinger ML, Genco RJ, Glogauer M, Goldstein M, Griffin TJ, Holmstrup P, Johnson GK, Kapila Y, Lang NP, Meyle J, Murakami S, Plemons J, Romito GA, Shapira L, Tatakis DN, Teughels W, Trombelli L, Walter C, Wimmer G, Xenoudi P, Yoshie H. Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: Consensus report of workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. J Clin Periodontol. 2018 Jun;45 Suppl 20:S68-S77. doi: 10.1111/jcpe.12940. PubMed PMID: 29926499.
15. Chen, H. W., W. Zhou, Y. Liao, S. C. Hu, T. L. Chen and Z. C. Song (2018). "Analysis of metabolic profiles of generalized aggressive periodontitis." J Periodontal Res 53(5): 894-901.
16. Chong J, Wishart DS, Xia J. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. Curr Protoc Bioinformatics. 2019 Dec;68(1): e86. doi: 10.1002/cpbi.86. PubMed PMID: 31756036.
17. Citterio F, Romano F, Meoni G, Iaderosa G, Grossi S, Sobrero A, Dego F, Corana M, Berta GN, Tenori L, Aimetti M. Changes in the Salivary Metabolic Profile of Generalized Periodontitis Patients after Non-surgical Periodontal Therapy: A Metabolomic Analysis Using Nuclear Magnetic Resonance Spectroscopy. J Clin Med. 2020 Dec 8;9(12):3977. doi: 10.3390/jcm9123977. PMID: 33302593; PMCID: PMC7763572.
18. Contrepois K, Jiang L, Snyder M. Optimized Analytical Procedures for the Untargeted Metabolomic Profiling of Human Urine and Plasma by Combining Hydrophilic Interaction (HILIC) and Reverse-Phase Liquid Chromatography(RPLC)-Mass Spectrometry. Mol Cell Proteomics. 2015 Jun;14(6):1684-95. doi:10.1074/mcp.M114.046508. Epub 2015 Mar 18. PubMed PMID: 25787789; PubMed Central PMCID: PMC4458729.
19. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N,Brown M, Knowles JD, Halsall A, Haselden JN, Nicholls AW, Wilson ID, Kell DB, Goodacre R; Human Serum Metabolome (HUSERMET) Consortium. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat Protoc. 2011 Jun 30;6(7):1060-83. doi: 10.1038/nprot.2011.335. PubMed PMID: 21720319.
20. Dunn WB, Wilson ID, Nicholls AW, Broadhurst D. The importance of experimental design and QC samples in large-scale and MS-driven untargeted metabolomic studies of humans. Bioanalysis. 2012 Sep;4(18):2249-64. doi: 10.4155/bio.12.204. Review. PubMed PMID: 23046267.
21. Elabdeen HR, Mustafa M, Szklenar M, Ruhl R, Ali R, Bolstad AI. Ratio of pro-resolving and pro-inflammatory lipid mediator precursors as potential markers for aggressive periodontitis. PLoS ONE. 2013;8:e70838.
22. Emwas, A. H. (2015). "The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research." Methods Mol Biol 1277: 161-193.
23. Fornasaro S , Berton F , Stacchi C , Farina F , Esposito A , Sergo V , Di Lenarda R , Bonifacio A . Label-free analysis of gingival crevicular fluid (GCF) by surface enhanced Raman scattering (SERS). Analyst. 2021 Feb 21;146(4):1464-1471. doi: 10.1039/d0an01997f. Epub 2021 Jan 11. PMID: 33427826.
24. Gawron K, Wojtowicz W, Łazarz-Bartyzel K, Łamasz A, Qasem B, Mydel P, Chomyszyn-Gajewska M, Potempa J, Mlynarz P. Metabolomic Status of The Oral Cavity in Chronic Periodontitis. In Vivo. 2019 Jul-Aug;33(4):1165-1174. doi:10.21873/invivo.11587

25. Golub LM, Kleinberg I. Gingival crevicular fluid: a new diagnostic aid in managing the periodontal patient. Oral Sci Rev. 1976;(8):49-61.
26. Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, Ojima Y, Tanaka K, Tanaka S, Aoshima K, Oda Y, Kakazu Y, Kusano M, Tohge T, Matsuda F, Sawada Y, Hirai MY, Nakanishi H, Ikeda K, Akimoto N, Maoka T, Takahashi H, Ara T, Sakurai N, Suzuki H, Shibata D, Neumann S, Iida T, Tanaka K, Funatsu K, Matsuura F, Soga T, Taguchi R, Saito K, Nishioka T. MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrom. 2010 Jul;45(7):703-14. doi: 10.1002/jms.1777. PubMed PMID: 20623627.
27. Kuboniwa, M., A. Sakanaka, E. Hashino, T. Bamba, E. Fukusaki and A. Amano (2016). "Prediction of Periodontal Inflammation via Metabolic Profiling of Saliva." J Dent Res 95(12): 1381-1386.
28. Liebsch C, Pitchika V, Pink C, Samietz S, Kastenmüller G, Artati A, Suhre K, Adamski J, Nauck M, Völzke H, Friedrich N, Kocher T, Holtfreter B, Pietzner M. The Saliva Metabolome in Association to Oral Health Status. J Dent Res. 2019 Jun;98(6):642-651. doi: 10.1177/0022034519842853. Epub 2019 Apr 26.
29. Morelli T, Agler CS, Divaris K. Genomics of periodontal disease and tooth morbidity. Periodontol 2000. 2020 Feb;82(1):143-156. doi: 10.1111/prd.12320. Review
30. Newbrun E. Indices to measure gingival bleeding. J Periodontol. 1996 Jun;67(6):555-61. Review. PubMed PMID: 8794964.
31. Okuda S, Yamada T, Hamajima M, Itoh M, Katayama T, Bork P, Goto S, Kanehisa M. KEGG Atlas mapping for global analysis of metabolic pathways. Nucleic Acids Res. 2008 Jul 1;36(Web Server issue):W423-6. doi: 10.1093/nar/gkn282. Epub 2008 May 13. PubMed PMID: 18477636; PubMed Central PMCID: PMC2447737.
32. O'Leary TJ, Drake RB, Naylor JE. The plaque control record. J Periodontol. 1972 Jan;43(1):38. PubMed PMID: 4500182.
33. Ozeki, M., T. Nozaki, J. Aoki, T. Bamba, K. R. Jensen, S. Murakami and M. Toyoda (2016). "Metabolomic Analysis of Gingival Crevicular Fluid Using Gas Chromatography/Mass Spectrometry." Mass Spectrom (Tokyo) 5(1): A0047.
34. Papapanou, P. N., M. Sanz, N. Buduneli, T. Dietrich, M. Feres, D. H. Fine, T. F. Flemmig, R. Garcia, W. V. Giannobile, F. Graziani, H. Greenwell, D. Herrera, R. T. Kao, M. Kebschull, D. F. Kinane, K. L. Kirkwood, T. Kocher, K. S. Kornman, P. S. Kumar, B. G. Loos, E. Machtei, H. Meng, A. Mombelli, I. Needleman, S. Offenbacher, G. J. Seymour, R. Teles and M. S. Tonetti (2018). "Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions." J
35. Pei J, Li F, Xie Y, Liu J, Yu T, Feng X. Microbial and metabolomic analysis of gingival crevicular fluid in general chronic periodontitis patients: lessons for a predictive, preventive, and personalized medical approach. EPMA J. 2020 Apr 16;11(2):197-215. doi: 10.1007/s13167-020-00202-5. PMID: 32547651; PMCID: PMC7272536.
36. Pluskal T, Castillo S, Villar-Briones A, Oresic M. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinformatics. 2010 Jul 23;11:395. doi: 10.1186/1471-2105-11-395. PubMed PMID: 20650010; PubMed Central PMCID: PMC2918584.
37. Ramfjord, S. P. (1967). "The Periodontal Disease Index (PDI)." J Periodontol 38(6): Suppl:602-610.
38. R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.
39. Righolt, A. J., M. Jevdjevic, W. Marcenes and S. Listl (2018). "Global-, Regional-, and Country-Level Economic Impacts of Dental Diseases in 2015." J Dent Res 97(5): 501-507.
40. Rohart F, Gautier B, Singh A, and Le Cao K-A (2017) mixOmics: An R package for 'omics feature selection and multiple data integration. PLoS computational biology 13(11):e1005752.
41. Romano, F., G. Meoni, V. Manavella, G. Baima, L. Tenori, S. Cacciatore and M. Aimetti (2018). "Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics." J Periodontol 89(12): 1452-1460.
42. Romano, F., G. Meoni, V. Manavella, G. Baima, G. M. Mariani, S. Cacciatore, L. Tenori and M. Aimetti (2019). "Effect of non-surgical periodontal therapy on salivary metabolic fingerprint of generalized chronic periodontitis using nuclear magnetic resonance spectroscopy." Arch Oral Biol 97: 208-214.

43. Sakanaka, A., et al. (2017). "Distinct signatures of dental plaque metabolic byproducts dictated by periodontal inflammatory status." Sci Rep 7: 42818.
44. Sanz M, Lau L, Herrera D, Morillo JM, Silva A. Methods of detection of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythensis in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. J Clin Periodontol. 2004 Dec;31(12):1034-47. doi: 10.1111/j.1600-051X.2004.00609.x. PMID: 15560803.
45. Smith, Q. T. (1977). "Gingival crevicular fluid as a diagnostic aid." Northwest Dent 56(2): 71-75.
46. Smith, Q. T. and S. J. Geegan (1991). "Repeated measurement of crevicular fluid parameters at different sites." J Clin Periodontol 18(3): 171-176.
47. Smith, Q. T., G. S. Au, P. L. Freese, J. B. Osborn and J. L. Stoltenberg (1992). "Five parameters of gingival crevicular fluid from eight surfaces in periodontal health and disease." J Periodontal Res 27(5): 466-475.
48. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem. 2006 Feb 1;78(3):779-87. PubMed PMID: 16448051.
49. Stein S. Mass spectral reference libraries: an ever-expanding resource for chemical identification. Anal Chem. 2012 Sep 4;84(17):7274-82. doi: 10.1021/ac301205z. Epub 2012 Jul 13. PubMed PMID: 22803687.
50. Takahashi N, Washio J, Mayanagi G. Metabolomics of supragingival plaque and oral bacteria. J Dent Res. 2010;89:1383-1388
51. Tonetti MS, Greenwell H, Kornman KS. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. J Periodontol. 2018 Jun;89 Suppl 1:S159-S172. doi: 10.1002/JPER.18-0006. Review. Erratum in: J Periodontol. 2018 Dec;89(12):1475.
52. Trindade, F., F. G. Oppenheim, E. J. Helmerhorst, F. Amado, P. S. Gomes and R. Vitorino (2014). "Uncovering the molecular networks in periodontitis." Proteomics Clin Appl 8(9-10): 748-761.
53. Veenstra, T. D. (2012). "Metabolomics: the final frontier?" Genome Med 4(4): 40.
54. Watts TL, Beards Cf, Ewing PD, Leeman S. Periodontal disease activity: a development strategy for its investigation by means of accurate 3-dimensional clinical measurement. J Clin Periodontol. 1995 Mar;22(3):201-7. doi: 10.1111/j.1600-051x.1995.tb00135.x. PMID: 7790525.
55. Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, Psychogios N, Dong E, Bouatra S, Mandal R, Sinelnikov I, Xia J, Jia L, Cruz JA, Lim E, Sobsey CA, Shrivastava S, Huang P, Liu P, Fang L, Peng J, Fradette R, Cheng D, Tzur D, Clements M, Lewis A, De Souza A, Zuniga A, Dawe M, Xiong Y, Clive D, Greiner R, Nazyrova A, Shaykhutdinov R, Li L, Vogel HJ, Forsythe I. HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 2009 Jan;37(Database issue):D603-10. doi: 10.1093/nar/gkn810. Epub 2008 Oct 25. PubMed PMID: 18953024; PubMed Central PMCID: PMC2686599.

III. Extended Results

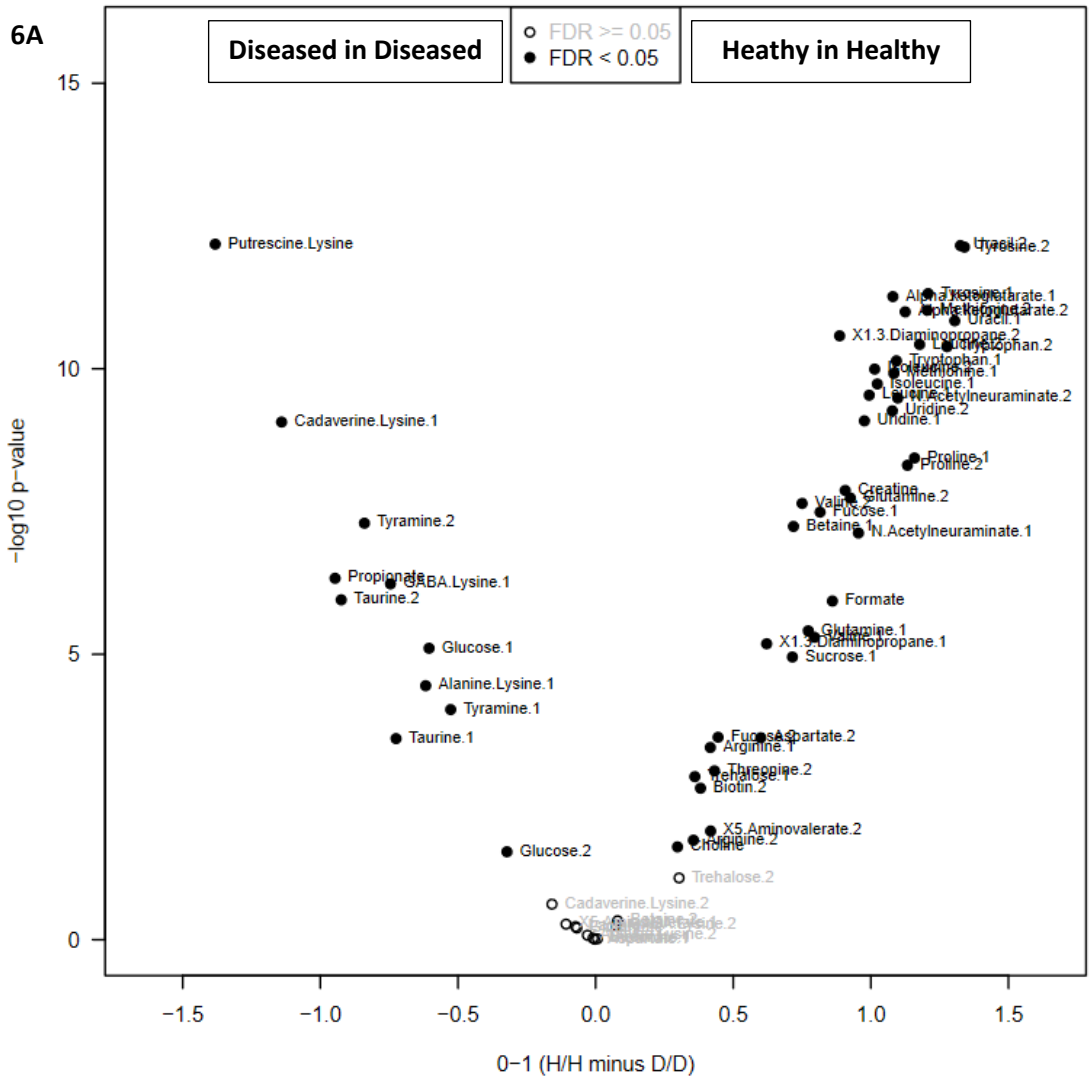
This project is part of an ongoing investigation focusing on the metabolites identified from the GCF in a site-specific manner. The subsequently presented results are a "pilot" presentation of the potential that MS spectrometry gives to this investigation. These results are deriving from analysis through MS of ten patients diagnosed with Stage III or IV periodontitis (test) and ten healthy controls and are analyzed only cross-sectionally.

Additionally, as explained in the protocol of our study, these patients, along with 30 more test and 30 more control subjects, are currently in 6-month follow-up protocol visit for re-sampling for a total of two years. It is expected that after MS and NMR the total sum of these samples is going to undergo the previously described longitudinal comparisons.

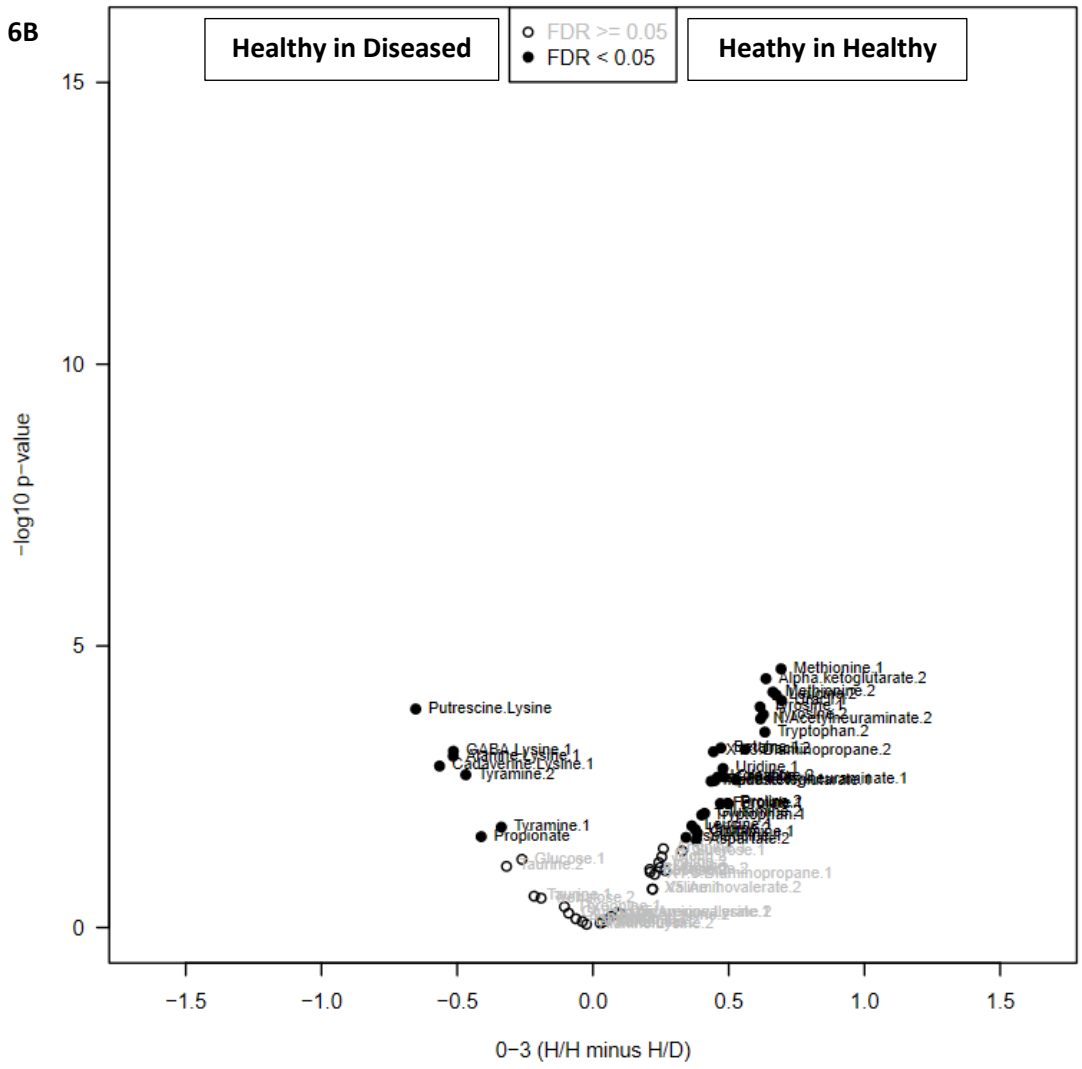
In a similarly designed, unpublished study by the same group, using just NMR the number of metabolites that were identified was 35. As expected, the much more sensitive MS detected a much larger number of metabolites in this branch of the study. However, there are results from this study that merit mention in our discussion.

Using data obtained from NMR analysis, we compared healthy sites in healthy subjects vs. diseased sites in diseased subjects, or healthy sites in healthy subjects' disease vs. healthy sites in diseased subjects and diseased sites in diseased subjects vs. healthy sites in diseased subjects (**Fig. 6**). As expected, the biggest differences are represented in the comparison between healthy sites of healthy subjects vs. diseased sites in diseased patients. The confidence in these results is quite high, with the FDR set 0.05. Interestingly, healthy

sites in healthy patient did not show significant differences when compared to healthy sites in diseased subjects.



6B


Healthy in Diseased
FDR >= 0.05
FDR < 0.05
Heathy in Healthy
15
10
5
0
−log10 p−value
−1.5
−1.0
−0.5
0.0
0.5
1.0
1.5
0−3 (H/H minus H/D)
Putrescine.Lysine
Tyramine.2
Tyramine.1
Propionate
Methionine.1
Alpha.ketoglutarate.2
N.Acetylneuraminate.2
Tryptophan.2
Uridine.1

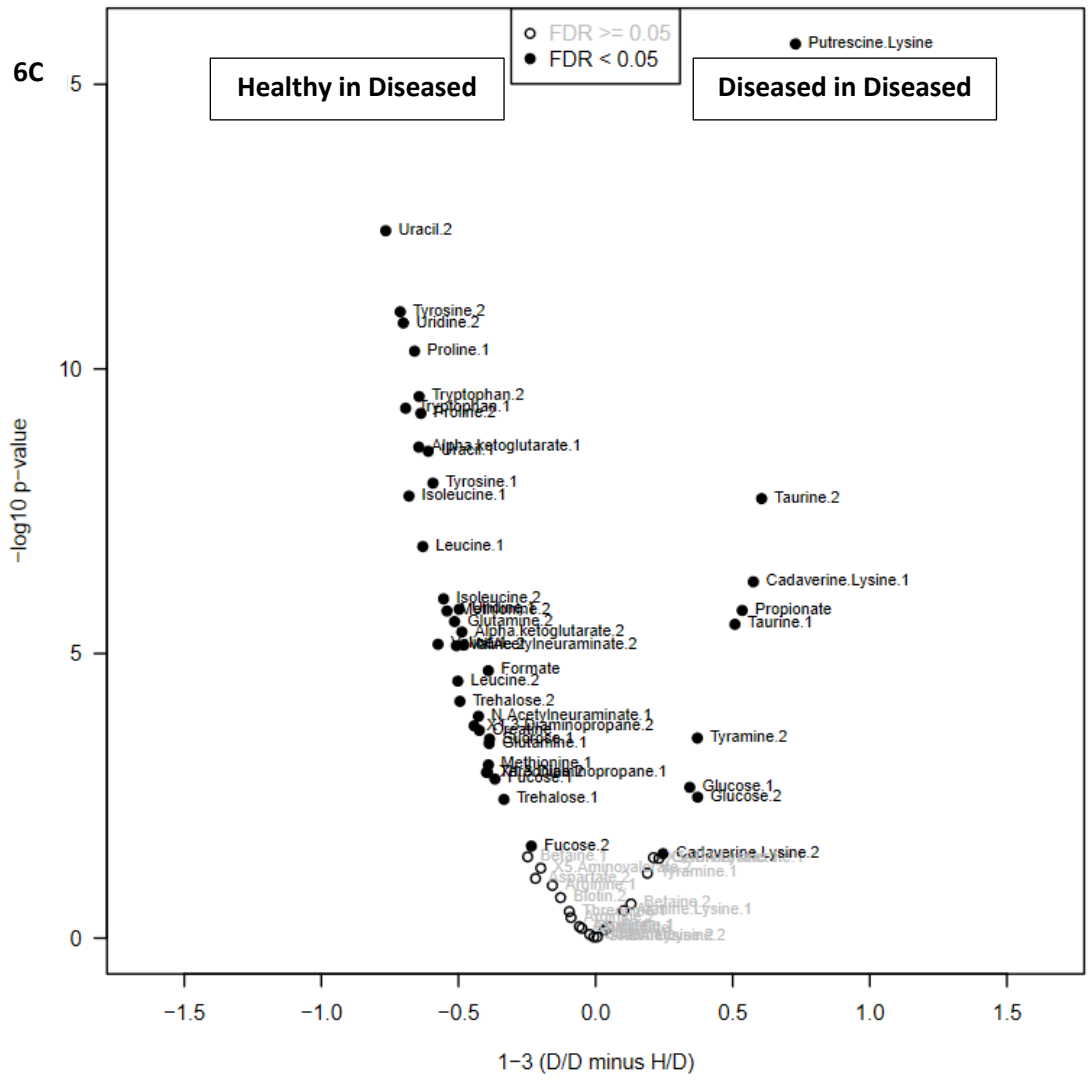


FIGURE 6. Volcano Plots of the baseline cross-sectional analysis by NMR **6A** Comparison of H/H vs. D/D. **6B** Comparison of H/H vs. H/D. **6C** Comparison of D/D vs. H/D

In the volcano plots each compound is represented by a single, dot. The dots are colored black if the FDR is <5% and white if FDR >5%. The identified metabolites have their names written by their assigned dot.

(H/H): healthy sites in healthy subjects

(D/D): diseased sites in diseased subjects

(H/D): healthy sites in diseased subjects

Furthermore, the yearly changes in metabolites over time with 95% confidence intervals were assessed and demonstrated in treated and untreated sites (**Fig. 7**). This comparison was made to represent the changes in the metabolites before and after periodontal treatment. The 95% confidence intervals for untreated are wide, potentially due to the small number of untreated patients in this group of that study, which does not allow for meaningful conclusions. The few untreated sites, also possibly due to the small sample of patients, led to a high FDR.

On the contrary, among the treated sites it is notable that decreases are observed in metabolites associated with periodontitis that were found in the baseline analysis (taurine, glucose, putrescine-lysine coherent resonances, cadaverine-lysine coherent resonances and propionate) and increases among some metabolites associated with health (uracil, creatine, methionine, isoleucine, aspartate, threonine, valine, arginine, trehalose). This means that, with treatment, metabolites that are associated with health increase, while metabolites associated with disease decrease.

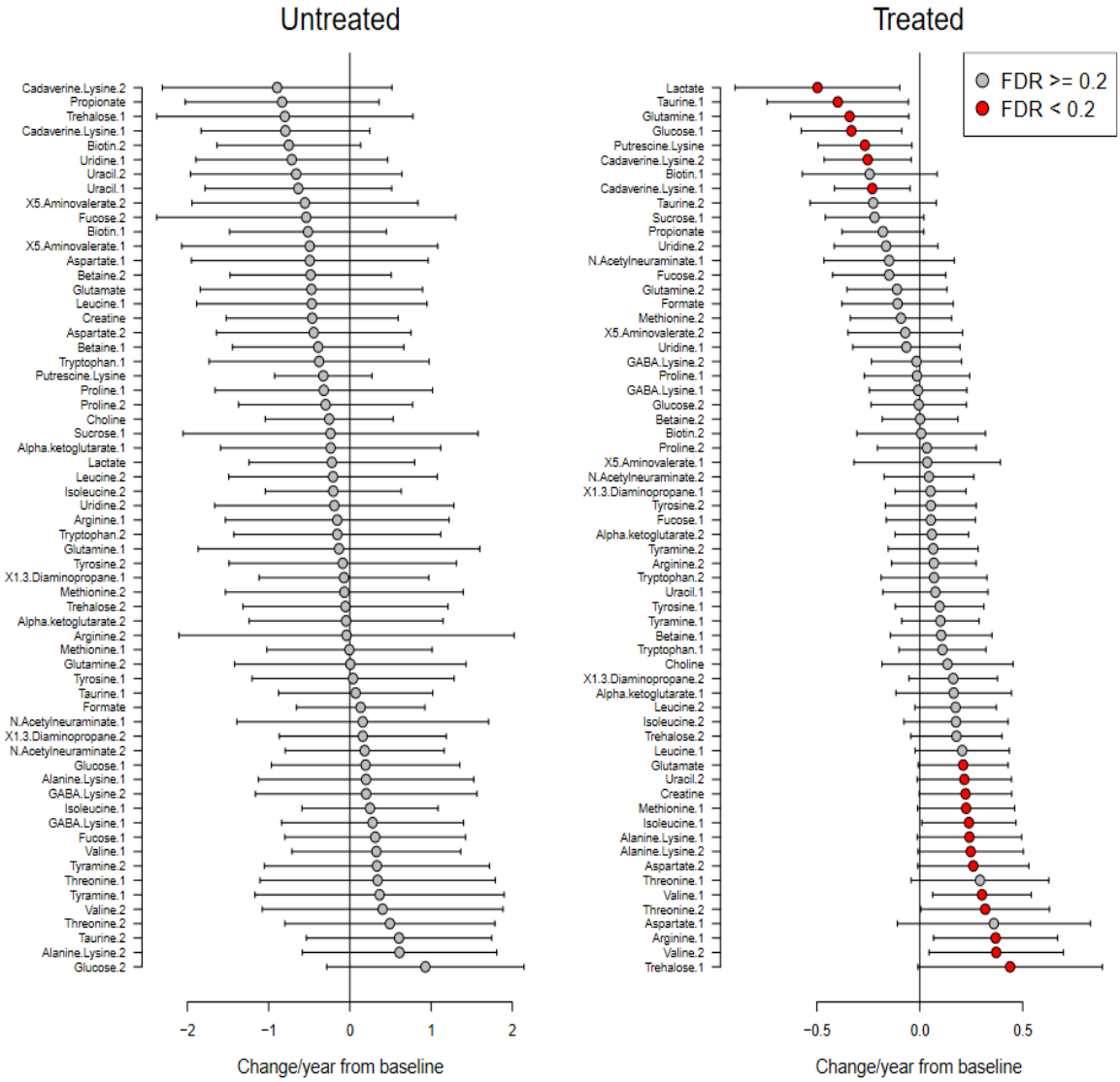


FIGURE 7 Longitudinal (yearly) changes in metabolites with 95% confidence interval. Left column presents the untreated sites and the right column presents the treated sites. These results have been a part of the NMR analysis. The dots are colored red if the FDR is <20% and grey if FDR >20%.

(H/H): healthy sites in healthy subjects

(D/D): diseased sites in diseased subjects

(H/D): healthy sites in diseased subjects

IV. Extended Discussion

Periodontitis is currently diagnosed and measured using the periodontal probe, a rudimentary and inaccurate in its measurements instrument (**Armitage 2003, Listgarten 1980, Isidor 1984, Van der Velden 1979-80**). Within its disadvantages, the periodontal probe can only discover the existence and history of periodontitis after it is established. The use of current technological advances and particularly the field of "-omics" are showing great potential to develop a diagnostic and prognostic test in the future for periodontitis. The focus of the literature is currently on the field of metabolomics (**Amberg 2017**).

As the technologies of NMR and MS in metabolomics has just recently advanced, there is lack of studies in the published literature, particularly in GCF (**Veentsra 2012, Emwas 2015, Schirra 2016, Barnes 2020**). Currently, while there are more studies on other biologic fluids, such as saliva and serum, as well as studies on plaque, there are not enough studies on GCF yet. The majority of available studies is on salivary metabolites (**Takahashi 2010, Elabdeen 2020, Mikkonen 2016, Sakanaka 2017, Chen 2018**).

Cadaverine, 5-oxoproline, and histidine have been shown to be metabolites isolated in saliva that are associated with periodontitis (**Kuboniwa 2016**). In the present study, cadaverine isolated from GCF samples is discovered with RPLC-MS and is positively associated with disease. Similarly, from our results from NMR analysis, cadaverine-lysine coherent resonances are associated with disease from cross-sectional data and gets reduced when periodontal therapy is performed.

Other identified salivary metabolites associated with periodontal disease are the dipeptides leucylisoleucine, phenylphenol, and serylisoleucine as well as the fatty acids arachidonate, arachidate, and dihomo-linolate are attractive candidate markers (**Barnes 2011**). The bacterial metabolite phenylacetate isolated from saliva has been significantly associated with periodontitis as well, making a potential biomarker for periodontitis (**Liebsch 2019**). These compounds have not been identified through NMR in our project. It is possible that they have been detected through MS and remained unidentified; a targeted approach is required to confirm the presence or absence of such compounds in the GCF.

A recent systematic review on salivary metabolomics concluded that valine, phenylalanine, isoleucine, tyrosine and butyrate are increased in subjects with periodontitis (**Baima 2021**). Furthermore, that same study, found that lactate, pyruvate and N-acetyl groups were increased in periodontal health. There are multiple contradictions among our results and the results of the Baima et. al. group, which could be attributed to the fact that our study's results come from GCF, while the Baima et. al. systematic review presents results of saliva metabolites. In contradiction with these results, butyric acid is positively associated with health and not periodontitis. In contrast to the results of this study, our GCF NMR-based project positively associated valine with health, rather than disease. Valine GCF levels identified through MS showed contradicting results among the different groups comparisons, being positively associated with both health and disease, while having a higher FDR. Regarding the N-acetyl groups, the NMR component of our research shows a positive association of N-Acetylneuraminate with health, in agreement with the research group results by Baima et. al. In yet another difference with the results obtained from

Baima et. al., while our MS results on GCF isoleucine levels were again contradictory among different groups comparisons, and linked to an FDR >20%, NMR managed to show a positive association with health. Regarding tyrosine, NMR showed increased tyrosine levels in healthy sites; however, MS contradicted these results by positively associating tyrosine with periodontitis, albeit with a higher FDR. Additionally, MS analysis in GCF samples showed a weak association of phenylalanine with disease, on par with the results of Baima et. al. It is interesting to note that, in our study, GCF sample NMR analysis, detected significant lactate reduction in subjects after treatment of periodontitis, while isoleucine and valine levels seemed to increase after treatment.

Additionally, the salivary metabolic fingerprints, through NMR, of chronic and aggressive periodontitis have been studied with no significant differences between the two conditions (**Romano 2018**). Confirming the results of the previous study, the profile of these two expressions of the disease shows increased proline, phenylalanine, and tyrosine and decreased levels of pyruvate, N-acetyl groups and lactate. Apart from the other metabolites that were previously discussed, proline as part of periodontal disease profile is confirmed by the MS results of our study. Surprisingly, NMR results strongly associates proline with periodontal health. Similar results from the same group are reported in a preceding study: acetate, c-aminobutyrate, n-butyrate, succinate, trimethylamine, propionate, phenylalanine and valine, are positively associated chronic periodontitis, while pyruvate and N-acetyl groups were again positively associated with periodontal health (**Aimetti 2012**). Our results from GCF NMR analysis confirm the propionate increased levels in disease status. Furthermore, an NMR-based study showed that non-surgical periodontal treatment is able to lead to significant changes in the salivary metabolic profile

of a periodontitis patient, however it will still be distinct from the one of a healthy patient (**Romano 2019, Citterio 2020**).

Another study managed to significantly relate periodontitis with the following metabolites in saliva: caproate, isocaproate/butyrate, isovalerate, isoleucine, isopropanol/methanol, 4-aminobutyrate, choline, sucrose, sucrose/glucose/lysine, lactate/proline, lactate and proline (**Garcia-Villaescusa 2018**). Of these metabolites, choline-related results from our NMR and MS studies are inconclusive on GCF. As far as sucrose, our NMR study has associated it with health, rather than disease, contradicting the above results.

Tongue swabs and saline washouts have been also able to identify metabolites that are related to health and disease through NMR analysis (**Gawron 2019**). Tongue swabs revealed increased levels of isopropanol and reduced levels of glycerol in periodontitis subjects. Saline washouts revealed increased levels of lactate and decreased levels of acetone and methanol in periodontitis subjects. While the association identified for lactate in our study is previously mentioned in the manuscript, none of the other compounds identified by Gawron et.al could be identified by our study. Looking at results from another group, short chain fatty acids, such as butyrate were found increased in the saliva of patients with periodontitis; however, lactate, γ-amino-butyrate, methanol, and threonine were decreased (**Rzenic 2017**). Having discussed all the previously-mentioned metabolites, threonine, in our NMR study was positively and significantly associated with health, and in addition to that, increased threonine levels were detected after successful periodontal treatment.

Despite the fact that saliva is more readily available compared to GCF, the latter has the advantage that it can reveal data on the metabolic profile on periodontitis in a site-specific manner. There is lack of literature currently on GCF metabolites associated with periodontitis. In a study that compared GCF obtained from healthy, gingivitis and periodontitis patients, the metabolic pathway of purine degradation, which is a major source for reactive oxygen species production, was significantly accelerated in periodontitis patients (**Barnes 2009**). Purine metabolism is a metabolic pathway that has been significantly associated with periodontitis, occurring from MS-analyzed data from our study.

When extracting data from studies that utilized metabolomic analysis of both GCF and serum, more metabolites could be identified through GCF (**Chen 2018**). In this study, both fluids could show significant differences between healthy and aggressive periodontitis patients. Focusing on the results from GCF, this study associated the following metabolites with generalized aggressive periodontitis: noradrenaline, uridine, α-tocopherol, dehydroascorbic acid, xanthine, galactose, glucose 1-phosphate and ribulose 5-phosphate. On the contrary, thymidine, glutathione and ribose 5-phosphate levels showed decrease in subjects with generalized aggressive periodontitis. Uridine has shown a trend for association with disease in the NMR branch our study, as it has increases in treated sites presenting with periodontal deterioration. Xanthine has been identified as a metabolite by MS in our study, however it presents inconclusive association with health or disease. D-Galactose has shown a strong association with disease in our study HILIC-MS analysis.

Metabolomic analysis through MS could be useful in prediction and diagnosis of the patient, showing distinct differences between healthy sites, shallower and deeper

pockets, which is promising for the development of a new test (**Ozeki 2016**). Putrescine, lysine, phenylalanine, ribose, taurine, 5-aminovaleric acid and galactose were identified to be higher in greater PDs. In our results from NMR, Putresine/Lysine coherent resonances were significantly associated with disease, while with treatment of the site, Putrecine/Lysine levels were significantly reduced. Additionally, cross-sectional data from MS confirm a trend for increase of putrescine in diseased site rather than the healthy sites of a diseased subject. MS results regarding lysine are inconclusive. As previously mentioned, MS results on phenylalanine show a weak trend for association with disease. While MS results on taurine are inconclusive, NMR showed a significant association with disease and a significant reduction in taurine after treatment of periodontitis, on par with the results of the above-mentioned study. This study by Ozeki et al. also showed a trend for increase on lactic acid, benzoic acid, glycine, malic acid, and phosphate with the increasing disease status of the patient. A similar trend for association with disease is also seen in our MS results for malic acid.

A recent systematic review and meta-analysis from 15 studies on GCF metabolites, 11 of which were targeted, managed to identify a total of 10 metabolites with available information regarding their association with gingival health, gingivitis or periodontitis (**Baima 2021**). This study concludes that oxidative stress-related metabolites are primarily reported in association with periodontitis. Malondialdehyde, 8-hydroxy-deoxyguanosine, 4-hydroxynonenal and neopterin were metabolite that has been associated with periodontitis. Conversely, glutathione was associated with periodontal health. None of these metabolites have been identified through the MS or NMR in our projects. However,

the targeted approach of MS analysis remains to be conducted, potentially revealing some associations with these metabolites.

A recent study reported on the discovery of GCF metabolites associated with chronic periodontitis (**Pei 2020**). Uracil, N-carbamylglutamate 2, N-acetyl-β-D-mannosamine 1, fructose 1, citramalic acid, 5-dihydrocortisol 3 and 4-hydroxyphenylacetic acid. thymidine 3 and O-phosphoserine 1 were associated with health. The result of uracil contradicts NMR results from our study, as it is mostly associated with health; additionally, we showed that uracil increase can lead to increase in improved clinical measurements. MS cross-sectional results on uracil have been inconclusive. None of the other compounds have yet been identified in our study, using either NMR or MS.

Analysis of GCF from periodontitis subjects using surface enhanced Raman scattering has led to the identification of uric acid, hypoxanthine, glutathione and ergothioneine, all of which were reduced in cases of periodontitis (**Fornasaro 2021**); however, uric acid results from our MS cross-sectional analysis are inconclusive.

The untargeted RPLC-MS and HILIC-MS results show the value of MS in the detection of numerous metabolites. From our analysis it was concluded that a targeted approach is essential to identify more metabolites with an FDR <5%, which will also enrich our existing library of metabolites for pathway analysis. From the analysis, not only with MS, but also with NMR, specific metabolites could be identified to be positively associated with either health or disease. In this discussion, the NMR and MS results come from a different sample of subjects, which makes the results from the two different methods not directly comparable. To overcome this, the same MS-analyzed sites presented in this

manuscript will be also be analyzed with NMR. Follow-up of the same subjects for 2 years will also give us longitudinal results.

V. References

1. Aimetti M, Cacciatore S, Graziano A, Tenori L. Metabonomic analysis of saliva reveals generalized chronic periodontitis signature. Metabolomics. 2012 Jun;8(3):465-74.
2. Amberg, A., B. Riefke, G. Schlotterbeck, A. Ross, H. Senn, F. Dieterle and M. Keck (2017). "NMR and MS Methods for Metabolomics." Methods Mol Biol 1641: 229-258.
3. Arias-Bujanda N, Regueira-Iglesias A, Balsa-Castro C, Nibali L, Donos N, Tomás I. Accuracy of single molecular biomarkers in gingival crevicular fluid for the diagnosis of periodontitis: A systematic review and meta-analysis. J Clin Periodontol. 2019 Dec;46(12):1166-1182. doi: 10.1111/jcpe.13188. Epub 2019 Oct 16. PMID: 31444912.
4. Arias-Bujanda N, Regueira-Iglesias A, Balsa-Castro C, Nibali L, Donos N, Tomás I. Accuracy of single molecular biomarkers in saliva for the diagnosis of periodontitis: A systematic review and meta-analysis. J Clin Periodontol. 2020 Jan;47(1):2-18. doi: 10.1111/jcpe.13202. Epub 2019 Nov 20. PMID: 31560804.
5. Armitage, G. C. (1999). "Development of a classification system for periodontal diseases and conditions." Ann Periodontol 4(1): 1-6.
6. Armitage, G. C., S. Research and P. Therapy Committee of the American Academy of (2003). "Diagnosis of periodontal diseases." J Periodontol 74(8): 1237-1247.
7. Aslam B, Basit M, Nisar MA, Khurshid M, Rasool MH. Proteomics: Technologies and Their Applications. J Chromatogr Sci. 2017 Feb;55(2):182-196. doi: 10.1093/chromsci/bmw167. Epub 2016 Oct 18. PMID: 28087761.
8. Baima G, Corana M, Iaderosa G, Romano F, Citterio F, Meoni G, Tenori L, Aimetti M. Metabolomics of gingival crevicular fluid to identify biomarkers for periodontitis: A systematic review with meta-analysis. J Periodontal Res. 2021 Mar 12. doi: 10.1111/jre.12872. Epub ahead of print. PMID: 33710624.
9. Baima G, Iaderosa G, Citterio F, Grossi S, Romano F, Berta GN, Buduneli N, Aimetti M. Salivary metabolomics for the diagnosis of periodontal diseases: a systematic review with methodological quality assessment. Metabolomics. 2021 Jan 1;17(1):1. doi: 10.1007/s11306-020-01754-3. PMID: 33387070.
10. Barnes VM, Teles R, Trivedi HM, Devizio W, Xu T, Mitchell MW, Milburn MV, Guo L. Acceleration of purine degradation by periodontal diseases. J Dent Res. 2009 Sep;88(9):851-5. doi: 10.1177/0022034509341967
11. Barnes, V. M., S. G. Ciancio, O. Shibly, T. Xu, W. Devizio, H. M. Trivedi, L. Guo and T. J. Jonsson (2011). "Metabolomics reveals elevated macromolecular degradation in periodontal disease." J Dent Res 90(11): 1293-1297.
12. Barnes S. Overview of Experimental Methods and Study Design in Metabolomics, and Statistical and Pathway Considerations. Methods Mol Biol. 2020;2104:1-10. doi: 10.1007/978-1-0716-0239-3_1. PMID: 31953809.
13. Barros, S. P., R. Williams, S. Offenbacher and T. Morelli (2016). "Gingival crevicular fluid as a source of biomarkers for periodontitis." Periodontol 2000 70(1): 53-64.

14. Douglas Bates, Martin Maechler, Ben Bolker, Steve Walker (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48. doi:10.18637/jss.v067.i01
15. Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. _Journal of the Royal Statistical Society Series B_, *57*, 289-300.
16. Bernini P, Bertini I, Luchinat C, Nincheri P, Staderini S, Turano P. Standard operating procedures for pre-analytical handling of blood and urine for metabolomic studies and biobanks. J Biomol NMR. 2011 Apr;49(3-4):231-43. doi: 10.1007/s10858-011-9489-1. Epub 2011 Mar 5. PubMed PMID: 21380509.
17. Bostanci, N. and G. N. Belibasakis (2018). "Gingival crevicular fluid and its immune mediators in the proteomic era." Periodontol 2000 76(1): 68-84.
18. Broadhurst D, Goodacre R, Reinke SN, Kuligowski J, Wilson ID, Lewis MR, Dunn WB. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. Metabolomics. 2018;14(6):72. doi: 10.1007/s11306-018-1367-3. Epub 2018 May 18. Review. PubMed PMID: 29805336; PubMed Central PMCID: PMC5960010.
19. Califf, K. J., K. Schwarzberg-Lipson, N. Garg, S. M. Gibbons, J. G. Caporaso, J. Slots, C. Cohen, P. C. Dorrestein and S. T. Kelley (2017). "Multi-omics Analysis of Periodontal Pocket Microbial Communities Pre- and Posttreatment." mSystems 2(3).
20. Cao, C. F. and Q. T. Smith (1989). "Crevicular fluid myeloperoxidase at healthy, gingivitis and periodontitis sites." J Clin Periodontol 16(1): 17-20.
21. Carneiro, L.G., Venuleo, C., Oppenheim, F.G. and Salih, E. (2012), Proteome data set of human gingival crevicular fluid from healthy periodontium sites by multidimensional protein separation and mass spectrometry. Journal of Periodontal Research, 47: 248-262. doi:10.1111/j.1600-0765.2011.01429.x
22. Chapple ILC, Mealey BL, Van Dyke TE, Bartold PM, Dommisch H, Eickholz P, Geisinger ML, Genco RJ, Glogauer M, Goldstein M, Griffin TJ, Holmstrup P, Johnson GK, Kapila Y, Lang NP, Meyle J, Murakami S, Plemons J, Romito GA, Shapira L, Tatakis DN, Teughels W, Trombelli L, Walter C, Wimmer G, Xenoudi P, Yoshie H. Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: Consensus report of workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. J Clin Periodontol. 2018 Jun;45 Suppl 20:S68-S77. doi: 10.1111/jcpe.12940. PubMed PMID: 29926499.
23. Chen, H. W., W. Zhou, Y. Liao, S. C. Hu, T. L. Chen and Z. C. Song (2018). "Analysis of metabolic profiles of generalized aggressive periodontitis." J Periodontal Res 53(5): 894-901.
24. Chong J, Wishart DS, Xia J. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. Curr Protoc Bioinformatics. 2019 Dec;68(1): e86. doi: 10.1002/cpbi.86. PubMed PMID: 31756036.
25. Citterio F, Romano F, Meoni G, Iaderosa G, Grossi S, Sobrero A, Dego F, Corana M, Berta GN, Tenori L, Aimetti M. Changes in the Salivary Metabolic Profile of Generalized Periodontitis Patients after Non-surgical Periodontal Therapy: A

Metabolomic Analysis Using Nuclear Magnetic Resonance Spectroscopy. J Clin Med. 2020 Dec 8;9(12):3977. doi: 10.3390/jcm9123977. PMID: 33302593; PMCID: PMC7763572.

26. Contrepois K, Jiang L, Snyder M. Optimized Analytical Procedures for the Untargeted Metabolomic Profiling of Human Urine and Plasma by Combining Hydrophilic Interaction (HILIC) and Reverse-Phase Liquid Chromatography(RPLC)-Mass Spectrometry. Mol Cell Proteomics. 2015 Jun;14(6):1684-95. doi:10.1074/mcp.M114.046508. Epub 2015 Mar 18. PubMed PMID: 25787789; PubMed Central PMCID: PMC4458729.
27. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N,Brown M, Knowles JD, Halsall A, Haselden JN, Nicholls AW, Wilson ID, Kell DB, Goodacre R; Human Serum Metabolome (HUSERMET) Consortium. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat Protoc. 2011 Jun 30;6(7):1060-83. doi: 10.1038/nprot.2011.335. PubMed PMID: 21720319.
28. Dunn WB, Wilson ID, Nicholls AW, Broadhurst D. The importance of experimental design and QC samples in large-scale and MS-driven untargeted metabolomic studies of humans. Bioanalysis. 2012 Sep;4(18):2249-64. doi: 10.4155/bio.12.204. Review. PubMed PMID: 23046267.
29. Ebersole, J. L., M. A. Taubman, D. J. Smith and J. M. Goodson (1984). "Gingival crevicular fluid antibody to oral microorganisms. I. Method of collection and analysis of antibody." J Periodontal Res 19(2): 124-132.
30. Ebersole, J. L., M. A. Taubman and D. J. Smith (1985). "Gingival crevicular fluid antibody to oral microorganisms. II. Distribution and specificity of local antibody responses." J Periodontal Res 20(4): 349-356.
31. Eke, P.I., Page, R.C., Wei, L., Thornton-Evans, G. and Genco, R.J. (2012), Update of the Case Definitions for Population-Based Surveillance of Periodontitis. Journal of Periodontology, 83: 1449-1454. doi:10.1902/jop.2012.110664
32. Eke PI, Thornton-Evans GO, Wei L, Borgnakke WS, Dye BA, Genco RJ. Periodontitis in US Adults: National Health and Nutrition Examination Survey 2009-2014. J Am Dent Assoc. 2018 Jul;149(7):576-588.e6. doi: 10.1016/j.adaj.2018.04.023
33. Elabdeen HR, Mustafa M, Szklenar M, Ruhl R, Ali R, Bolstad AI. Ratio of pro-resolving and pro-inflammatory lipid mediator precursors as potential markers for aggressive periodontitis. PLoS ONE. 2013;8:e70838.
34. Emwas, A. H. (2015). "The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research." Methods Mol Biol 1277: 161-193.
35. Flemmig, T. F. and T. Beikler (2013). "Economics of periodontal care: market trends, competitive forces and incentives." Periodontol 2000 62(1): 287-304
36. Fornasaro S , Berton F , Stacchi C , Farina F , Esposito A , Sergo V , Di Lenarda R , Bonifacio A . Label-free analysis of gingival crevicular fluid (GCF) by surface enhanced Raman scattering (SERS). Analyst. 2021 Feb 21;146(4):1464-1471. doi: 10.1039/d0an01997f. Epub 2021 Jan 11. PMID: 33427826.

37. García-Villaescusa A, Morales-Tatay JM, Monleón-Salvadó D, González-Darder JM, Bellot-Arcis C, Montiel-Company JM, Almerich-Silla JM. Using NMR in saliva to identify possible biomarkers of glioblastoma and chronic periodontitis. PLoS One. 2018 Feb 6;13(2):e0188710. doi: 10.1371/journal.pone.0188710. PMID: 29408884; PMCID: PMC5800567.
38. Gawron K, Wojtowicz W, Łazarz-Bartyzel K, Łamasz A, Qasem B, Mydel P, Chomyszyn-Gajewska M, Potempa J, Mlynarz P. Metabolomic Status of The Oral Cavity in Chronic Periodontitis. In Vivo. 2019 Jul-Aug;33(4):1165-1174. doi:10.21873/invivo.11587
39. Ghallab, N. A. (2018). "Diagnostic potential and future directions of biomarkers in gingival crevicular fluid and saliva of periodontal diseases: Review of the current evidence." Arch Oral Biol 87: 115-124.
40. Golub LM, Kleinberg I. Gingival crevicular fluid: a new diagnostic aid in managing the periodontal patient. Oral Sci Rev. 1976;(8):49-61.
41. Grant MM. What do 'omic technologies have to offer periodontal clinical practice in the future? J Periodontal Res. 2012 Feb;47(1):2-14. doi: 10.1111/j.1600-0765.2011.01387.x. Epub 2011 Jun 17. Review.
42. Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, Ojima Y, Tanaka K, Tanaka S, Aoshima K, Oda Y, Kakazu Y, Kusano M, Tohge T, Matsuda F, Sawada Y, Hirai MY, Nakanishi H, Ikeda K, Akimoto N, Maoka T, Takahashi H, Ara T, Sakurai N, Suzuki H, Shibata D, Neumann S, Iida T, Tanaka K, Funatsu K, Matsuura F, Soga T, Taguchi R, Saito K, Nishioka T. MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrom. 2010 Jul;45(7):703-14. doi: 10.1002/jms.1777. PubMed PMID: 20623627.
43. Huri, C. B., N. Yamalik, K. Kilinc, A. Kilinc, I. Etikan and K. Eratalay (2003). "Analysis of the relationship between the severity of periodontal destruction and proteoglycan metabolism of gingiva and gingival crevicular flu." J Clin Periodontol 30(11): 961-968.
44. Isidor F, Karring T, Attström R. Reproducibility of pocket depth and attachment level measurements when using a flexible splint. *J Clin Periodontol*. 1984;11(10):662-668. doi:10.1111/j.1600-051X.1984.tb01314.x
45. Kassebaum, N. J., E. Bernabe, M. Dahiya, B. Bhandari, C. J. Murray and W. Marcenes (2014). "Global burden of severe periodontitis in 1990-2010: a systematic review and meta-regression." J Dent Res 93(11): 1045-1053
46. Kc S, Wang XZ, Gallagher JE. Diagnostic sensitivity and specificity of host-derived salivary biomarkers in periodontal disease amongst adults: Systematic review. J Clin Periodontol. 2020 Mar;47(3):289-308. doi: 10.1111/jcpe.13218. Epub 2019 Dec 26. PMID: 31701554.
47. Kido, J., Bando, M., Hiroshima, Y., Iwasaka, H., Yamada, K., Ohgami, N., Nambu, T., Kataoka, M., Yamamoto, T., Shinohara, Y., Sagawa, I. and Nagata, T. (2012), Analysis of proteins in human gingival crevicular fluid by mass spectrometry. Journal of Periodontal Research, 47: 488-499. doi:10.1111/j.1600-0765.2011.01458.x
48. Kinney, J. S., T. Morelli, M. Oh, T. M. Braun, C. A. Ramseier, J. V. Sugai and W. V. Giannobile (2014). "Crevicular fluid biomarkers and periodontal disease progression." J Clin Periodontol 41(2): 113-120.

49. Kuboniwa, M., A. Sakanaka, E. Hashino, T. Bamba, E. Fukusaki and A. Amano (2016). "Prediction of Periodontal Inflammation via Metabolic Profiling of Saliva." J Dent Res 95(12): 1381-1386.
50. Kwok V, Caton JG. Commentary: prognosis revisited: a system for assigning periodontal prognosis. J Periodontol. 2007 Nov;78(11):2063-71. Review. PubMed PMID: 17970671
51. Lang NP, Adler R, Joss An, Nyman S; Absence of bleeding on probing: An indicator of periodontal stability; Journal of Clinical Periodontology. 1990
52. Leppilahti, J. M., T. Sorsa, M. A. Kallio, T. Tervahartiala, G. Emingil, B. Han and P. Mantyla (2015). "The utility of gingival crevicular fluid matrix metalloproteinase-8 response patterns in prediction of site-level clinical treatment outcome." J Periodontol 86(6): 777-787.
53. Liebsch C, Pitchika V, Pink C, Samietz S, Kastenmüller G, Artati A, Suhre K, Adamski J, Nauck M, Völzke H, Friedrich N, Kocher T, Holtfreter B, Pietzner M. The Saliva Metabolome in Association to Oral Health Status. J Dent Res. 2019 Jun;98(6):642-651. doi: 10.1177/0022034519842853. Epub 2019 Apr 26.
54. Listgarten MA. Periodontal probing: What does it mean? J Clin Periodontol. 1980;7:165-176.
55. Martin JA, Page RC, Kaye EK, Hamed MT, Loeb CF. Periodontitis severity plus risk as a tooth loss predictor. J Periodontol. 2009 Feb;80(2):202-9. doi:10.1902/jop.2009.080363
56. McGuire MK, Nunn ME; Prognosis versus actual outcome II. The effectiveness of clinical parameters in developing an accurate prognosis; Journal of Periodontology 1966; Jul;67(7):658-65
57. Mikkonen JJ, Singh SP, Herrala M, Lappalainen R, Myllymaa S, Kullaa AM. Salivary metabolomics in the diagnosis of oral cancer and periodontal diseases. J Periodontal Res. 2016 Aug;51(4):431-7. doi: 10.1111/jre.12327. Epub 2015 Oct 8. PMID: 26446036.
58. Morelli T, Agler CS, Divaris K. Genomics of periodontal disease and tooth morbidity. Periodontol 2000. 2020 Feb;82(1):143-156. doi: 10.1111/prd.12320. Review
59. Newbrun E. Indices to measure gingival bleeding. J Periodontol. 1996 Jun;67(6):555-61. Review. PubMed PMID: 8794964.
60. Nunn ME, Fan J, Su X, Levine RA, Lee HJ, Mcguire MK. Development of prognostic indicators using classification and regression trees for survival. *Periodontol 2000*. 2012;58(1):134-142. doi:10.1111/j.1600-0757.2011.00421.x
61. Okuda S, Yamada T, Hamajima M, Itoh M, Katayama T, Bork P, Goto S, Kanehisa M. KEGG Atlas mapping for global analysis of metabolic pathways. Nucleic Acids Res. 2008 Jul 1;36(Web Server issue):W423-6. doi: 10.1093/nar/gkn282. Epub 2008 May 13. PubMed PMID: 18477636; PubMed Central PMCID: PMC2447737.
62. O'Leary TJ, Drake RB, Naylor JE. The plaque control record. J Periodontol. 1972 Jan;43(1):38. PubMed PMID: 4500182.
63. Ozeki, M., T. Nozaki, J. Aoki, T. Bamba, K. R. Jensen, S. Murakami and M. Toyoda (2016). "Metabolomic Analysis of Gingival Crevicular Fluid Using Gas Chromatography/Mass Spectrometry." Mass Spectrom (Tokyo) 5(1): A0047.

64. Papapanou, P. N., M. Sanz, N. Buduneli, T. Dietrich, M. Feres, D. H. Fine, T. F. Flemmig, R. Garcia, W. V. Giannobile, F. Graziani, H. Greenwell, D. Herrera, R. T. Kao, M. Kebschull, D. F. Kinane, K. L. Kirkwood, T. Kocher, K. S. Kornman, P. S. Kumar, B. G. Loos, E. Machtei, H. Meng, A. Mombelli, I. Needleman, S. Offenbacher, G. J. Seymour, R. Teles and M. S. Tonetti (2018). "Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions." J
65. Pei J, Li F, Xie Y, Liu J, Yu T, Feng X. Microbial and metabolomic analysis of gingival crevicular fluid in general chronic periodontitis patients: lessons for a predictive, preventive, and personalized medical approach. EPMA J. 2020 Apr 16;11(2):197-215. doi: 10.1007/s13167-020-00202-5. PMID: 32547651; PMCID: PMC7272536.
66. Pluskal T, Castillo S, Villar-Briones A, Oresic M. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinformatics. 2010 Jul 23;11:395. doi: 10.1186/1471-2105-11-395. PubMed PMID: 20650010; PubMed Central PMCID: PMC2918584.
67. Ramfjord, S. P. (1967). "The Periodontal Disease Index (PDI)." J Periodontol 38(6): Suppl:602-610.
68. R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.
69. Reinhardt, R. A., J. A. Stoner, L. M. Golub, H. M. Lee, P. V. Nummikoski, T. Sorsa and J. B. Payne (2010). "Association of gingival crevicular fluid biomarkers during periodontal maintenance with subsequent progressive periodontitis." J Periodontol 81(2): 251-259.
70. Righolt, A. J., M. Jevdjevic, W. Marcenes and S. Listl (2018). "Global-, Regional-, and Country-Level Economic Impacts of Dental Diseases in 2015." J Dent Res 97(5): 501-507.
71. Rohart F, Gautier B, Singh A, and Le Cao K-A (2017) mixOmics: An R package for 'omics feature selection and multiple data integration. PLoS computational biology 13(11):e1005752.
72. Romano, F., G. Meoni, V. Manavella, G. Baima, L. Tenori, S. Cacciatore and M. Aimetti (2018). "Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics." J Periodontol 89(12): 1452-1460.
73. Romano, F., G. Meoni, V. Manavella, G. Baima, G. M. Mariani, S. Cacciatore, L. Tenori and M. Aimetti (2019). "Effect of non-surgical periodontal therapy on salivary metabolic fingerprint of generalized chronic periodontitis using nuclear magnetic resonance spectroscopy." Arch Oral Biol 97: 208-214.
74. Rzeznik M, Triba MN, Levy P, Jungo S, Botosoa E, Duchemann B, Le Moyec L, Bernaudin JF, Savarin P, Guez D. Identification of a discriminative metabolomic fingerprint of potential clinical relevance in saliva of patients with periodontitis using 1H nuclear magnetic resonance (NMR) spectroscopy. PloS one. 2017 Aug 24;12(8):e0182767.
75. Sakanaka, A., et al. (2017). "Distinct signatures of dental plaque metabolic byproducts dictated by periodontal inflammatory status." Sci Rep 7: 42818.

76. Sanikop, S., S. Patil and P. Agrawal (2012). "Gingival crevicular fluid alkaline phosphatase as a potential diagnostic marker of periodontal disease." J Indian Soc Periodontol 16(4): 513-518.
77. Sanz M, Lau L, Herrera D, Morillo JM, Silva A. Methods of detection of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythensis in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. J Clin Periodontol. 2004 Dec;31(12):1034-47. doi: 10.1111/j.1600-051X.2004.00609.x. PMID: 15560803.
78. Schirra HJ, Ford PJ. NMR-Based Metabolomics of Oral Biofluids. Methods Mol Biol. 2017;1537:79-105. doi: 10.1007/978-1-4939-6685-1_5. PMID: 27924589.
79. Smith, Q. T. (1977). "Gingival crevicular fluid as a diagnostic aid." Northwest Dent 56(2): 71-75.
80. Smith, D. J., L. M. Gadalla, J. L. Ebersole and M. A. Taubman (1985). "Gingival crevicular fluid antibody to oral microorganisms. III. Association of gingival homogenate and gingival crevicular fluid antibody levels." J Periodontal Res 20(4): 357-367.
81. Smith, Q. T., J. E. Hinrichs and R. S. Melnyk (1986). "Gingival crevicular fluid myeloperoxidase at periodontitis sites." J Periodontal Res 21(1): 45-55.
82. Smith, Q. T. and S. J. Geegan (1991). "Repeated measurement of crevicular fluid parameters at different sites." J Clin Periodontol 18(3): 171-176.
83. Smith, Q. T., G. S. Au, P. L. Freese, J. B. Osborn and J. L. Stoltenberg (1992). "Five parameters of gingival crevicular fluid from eight surfaces in periodontal health and disease." J Periodontal Res 27(5): 466-475.
84. Smith, Q. T., Y. D. Wang and B. Sim (1994). "Inhibition of crevicular fluid neutrophil elastase by alpha 1-antitrypsin in periodontal health and disease." Arch Oral Biol 39(4): 301-306.
85. Smith, Q. T., L. Harriman, G. S. Au, J. L. Stoltenberg, J. B. Osborn, D. M. Aeppli and G. Fischer (1995). "Neutrophil elastase in crevicular fluid: comparison of a middle-aged general population with healthy and periodontitis groups." J Clin Periodontol 22(12): 935-941.
86. Smith, A. J., M. Addy and G. Embery (1995). "Gingival crevicular fluid glycosaminoglycan levels in patients with chronic adult periodontitis." J Clin Periodontol 22(5): 355-361.
87. Smith, A. J., W. Wade, M. Addy and G. Embery (1997). "The relationship between microbial factors and gingival crevicular fluid glycosaminoglycans in human adult periodontitis." Arch Oral Biol 42(1): 89-92.
88. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem. 2006 Feb 1;78(3):779-87. PubMed PMID: 16448051.
89. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998 Feb;25(2):134-44
90. Stadler, A. F., P. D. Angst, R. M. Arce, S. C. Gomes, R. V. Oppermann and C. Susin (2016). "Gingival crevicular fluid levels of cytokines/chemokines in chronic periodontitis: a meta-analysis." J Clin Periodontol 43(9): 727-745.

91. Stein S. Mass spectral reference libraries: an ever-expanding resource for chemical identification. Anal Chem. 2012 Sep 4;84(17):7274-82. doi: 10.1021/ac301205z. Epub 2012 Jul 13. PubMed PMID: 22803687.
92. Takahashi N, Washio J, Mayanagi G. Metabolomics of supragingival plaque and oral bacteria. J Dent Res. 2010;89:1383-1388
93. Teles, R., D. Sakellari, F. Teles, A. Konstantinidis, R. Kent, S. Socransky and A. Haffajee (2010). "Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota." J Periodontol 81(1): 89-98.
94. Tonetti MS, Greenwell H, Kornman KS. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. J Periodontol. 2018 Jun;89 Suppl 1:S159-S172. doi: 10.1002/JPER.18-0006. Review. Erratum in: J Periodontol. 2018 Dec;89(12):1475.
95. Trindade, F., F. G. Oppenheim, E. J. Helmerhorst, F. Amado, P. S. Gomes and R. Vitorino (2014). "Uncovering the molecular networks in periodontitis." Proteomics Clin Appl 8(9-10): 748-761.
96. Tsuchida, S., Satoh, M., Umemura, H., Sogawa, K., Kawashima, Y., Kado, S., Sawai, S., Nishimura, M., Kodera, Y., Matsushita, K. and Nomura, F. (2012), Proteomic analysis of gingival crevicular fluid for discovery of novel periodontal disease markers. Proteomics, 12: 2190-2202. doi:10.1002/pmic.201100655
97. Van der Velden U. Probing force and the relationship of the probe tip to the periodontal tissues. J Clin Periodontol. 1979;6:106-114.
98. Van der Velden U, de Vries J. The influence of probing force on the reproducibility of pocket depth measurements. J Clin Periodontol. 1980;7:414-420.
99. Veenstra, T. D. (2012). "Metabolomics: the final frontier?" Genome Med 4(4): 40.
100. Watts TL, Beards Cf, Ewing PD, Leeman S. Periodontal disease activity: a development strategy for its investigation by means of accurate 3-dimensional clinical measurement. J Clin Periodontol. 1995 Mar;22(3):201-7. doi: 10.1111/j.1600-051x.1995.tb00135.x. PMID: 7790525.
101. Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, Psychogios N, Dong E, Bouatra S, Mandal R, Sinelnikov I, Xia J, Jia L, Cruz JA, Lim E, Sobsey CA, Shrivastava S, Huang P, Liu P, Fang L, Peng J, Fradette R, Cheng D, Tzur D, Clements M, Lewis A, De Souza A, Zuniga A, Dawe M, Xiong Y, Clive D, Greiner R, Nazyrova A, Shaykhutdinov R, Li L, Vogel HJ, Forsythe I. HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 2009 Jan;37(Database issue):D603-10. doi: 10.1093/nar/gkn810. Epub 2008 Oct 25. PubMed PMID: 18953024; PubMed Central PMCID: PMC2686599.